

Thesis for the Master's degree in Biosciences
Main field of study in Molecular Biology

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Molecular mechanisms for
antibody-induced endocytosis
of ErbB2

60 study points

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AP	Adaptor protein
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CME	Clathrin-mediated endocytosis
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmatic reticulum
ESCRT	Endosomal sorting complex required for transport
FBS	Fetal bovine serum
GA	Geldanamycin
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HA	Human influenza hemagglutinin
HRP	Horseradish peroxidase
IP	Immunoprecipitation
mAb	monoclonal antibody
MEM	Minimum essential medium
MVB	Multivesicular body
NEM	N-ethylmaleimide
PAE	Porcine aortic endothelial
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
pY	phosphorylated tyrosine
RING	Really Interesting New Gene
RRX	Rhodamine Red-X
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate

SH2/3 Src homology 2/3

siRNA Small interfering RNA

TBST Tris-buffered saline with Tween20

TGF Transforming growth factor

TULA T-cell ubiquitin ligand

UBD Ubiquitin-binding domain

v/v Volume to volume

wt/v Weight to volume

Abstract

The four receptor tyrosine kinases of the ErbB family mediate the activation of a complex network of signalling pathways that regulate cell proliferation, differentiation, and survival. Because of their central role in the regulation of these cellular processes, the receptors are often found to have abnormal activity in cancer cells. This makes them important targets for cancer treatment. The ErbB proteins depend on dimerization to exert their function and they are generally downregulated through the internalization and degradation of activated receptors. ErbB2, however, is special in that it is resistant to internalization and inhibits internalization of other ErbBs upon heterodimerization. For this reason, therapeutic antibodies have been developed that block the receptors' ability to dimerize and/or induces their downregulation.

In the present study, two new therapeutic antibodies against ErbB2 are investigated. The study shows that they induce internalization and degradation of the receptor. Investigations into which endocytic pathway the cells use to internalize ErbB2 upon treatment with these antibodies reveal that the main pathway(s) used is/are most likely dynamin-dependent. Further, the study shows that the antibodies induce both phosphorylation and ubiquitination of the receptor. The activities of the new antibodies were also compared to those of the therapeutic antibodies trastuzumab and pertuzumab, which are already in clinical use, and the new antibodies appear to be more effective at inducing downregulation of ErbB2.

1 Introduction

1.1 Endocytic pathways

Endocytosis is the process by which material is engulfed by a cell and internalized. It is divided into two main categories; phagocytosis, which is the uptake of large particles that typically only happens in specialized cells, and pinocytosis, which is the uptake of fluids and soluble molecules. The latter is further divided into four pathways; macropinocytosis, clathrin-mediated endocytosis (CME), caveolin-mediated endocytosis (it is disputed whether this one actually exists), and clathrin- and caveolin-independent endocytosis (Figure 1). The best understood pathway is CME. It plays an important part in modulating signal transduction, as it controls the levels of receptors in the plasma membrane and mediate their downregulation when they are activated. CME mediates internalization of ligand-activated membrane receptors by concentrating them into clathrin-coated pits. Clathrin is a protein that assembles into a lattice-like structure, or cage, on the cytosolic side of the plasma membrane which causes membrane invagination. In order for the clathrin-cage to form, adaptor protein complexes (AP1-4) must first bind to the transmembrane receptors that are to be internalized. The APs are involved in vesicle formation in many locations inside the cell, but only AP2 mediates endocytosis. Once the coated pits have formed, the GTPase dynamin assembles around the “neck” of the invaginated membrane and hydrolysis of GTP induces a conformational change which pinches off the membrane and creates an intracellular vesicle containing the coated pit-associated receptors and their ligands. These vesicles then fuse with early endosomes and their contents are either recycled back to the plasma membrane or transported elsewhere inside the cell. While the function of dynamin is best understood in relation to its role in CME, it also has a similar function and is required for phagocytosis, caveolin-mediated endocytosis, and some clathrin- and caveolin-independent endocytic pathways (reviewed in *1*).

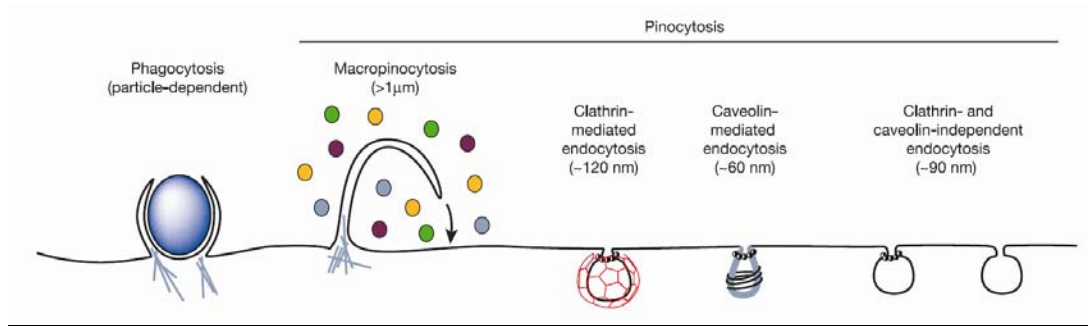


Figure 1: The endocytosis pathways. Endocytosis is accomplished through two main categories of pathway; phagocytosis and pinocytosis. Pinocytosis is further divided into macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin- and caveolin-independent endocytosis. The figure is taken from (1). The figure legend is modified.

1.2 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are a superfamily of cell-surface localized receptors involved in transduction of extracellular signals which trigger intracellular pathways that regulates important processes like proliferation and differentiation, survival and metabolism, cell migration, and cell-cycle control. All RTKs have similar structural elements; an extracellular region with ligand-binding domains, a single transmembrane α -helix, and an intracellular region with tyrosine kinase domain(s) as well as other regulatory domains. Binding of a ligand to RTKs causes them to dimerize, which induces their kinase activity where they phosphorylate themselves and other targets, leading to propagation of the signal downstream. Abnormalities in RTK function has been linked to several diseases, including many types of cancer (reviewed in 2).

1.3 ErbB family receptors

The ErbBs are a family of RTKs, the first of which, epidermal growth factor receptor (EGFR/ErbB1/HER1) was discovered and described by Nobel laureate Stanley Cohen as a result of his research on epidermal growth factor (EGF), the receptor's ligand (3). EGFR has been important in studying endocytic pathways. The mechanisms by which it is endocytosed are well understood and EGFR is used as a model for the endocytosis of other RTKs. The other ErbBs, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4, were discovered much later and are less well-characterized. Ligand binding induces both homo- and heterodimerization, each with their own unique properties providing a high degree of signaling diversity (4, 5).

1.3.1 Structure and function

The ErbB proteins all have the same general structure (Figure 2). The extracellular region consists of four domains numbered I-IV. Domains I and III are responsible for ligand binding, both making contact with the same ligand. Domain II, which forms the connection between domain I and III, contains a dimerization arm that gets exposed due to a conformational change from “closed”, where the arm is hidden by domain IV, to “open” being induced as the result of a ligand binding to the receptor. This change makes the receptor available for dimerization with other open ErbBs, or other RTKs such as MET (6, 7). The intracellular region of ErbBs includes a tyrosine kinase domain, with an N-terminal and C-terminal lobe, and a C-terminal tail containing many tyrosines that are possible phosphorylation sites. Upon dimerization, an asymmetrical interaction is formed between the intracellular tyrosine kinase domains of each receptor where the C-terminal lobe on one of the receptors makes contact with the N-terminal lobe of the other. This causes them to phosphorylate each other and thus activate (8).

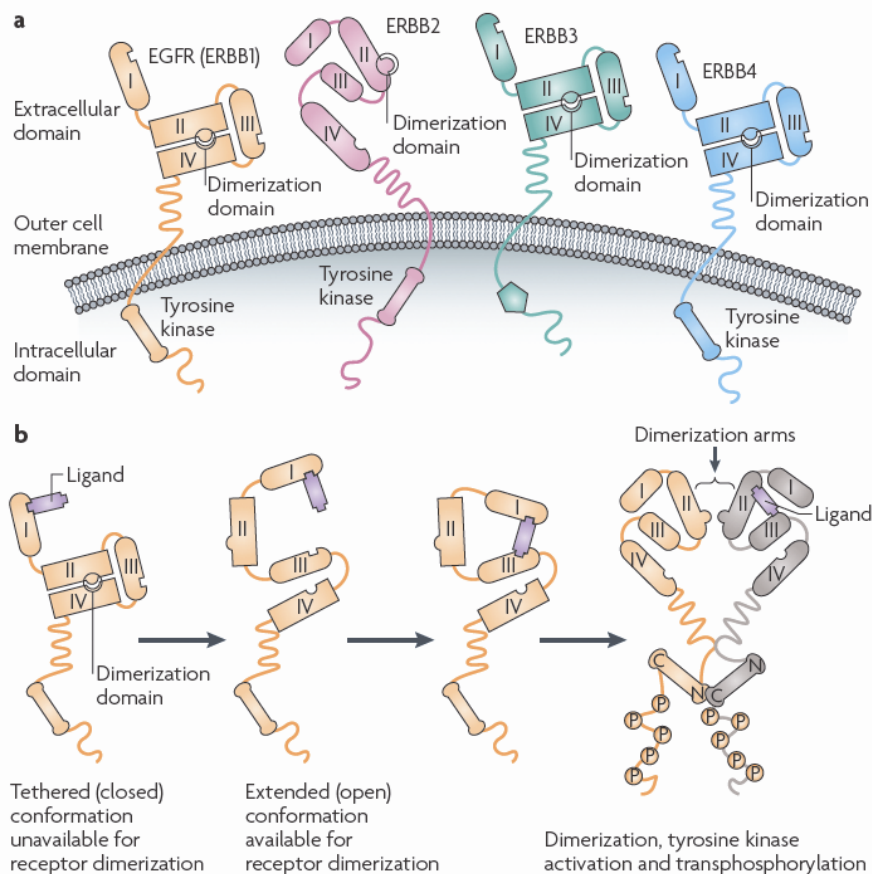


Figure 2: The structure and function of ErbBs. (a) The ErbB family proteins consist of a ligand-binding extracellular region, a transmembrane region, and an intracellular region with enzymatic activity and docking sites for other proteins. (b) When a ligand binds to the receptor, it changes conformation which allows it to form active dimers with other receptors. The figure is taken from (9). The figure legend is modified.

While the overall structures of the ErbBs are similar, there are important functional differences. EGFR and ErbB4 have several ligands and possess tyrosine kinase activity. ErbB3 has ligands but its kinase activity is impaired. ErbB2, on the other hand, has kinase activity, but no known ligand and exists in a perpetually open conformation (6, 10). ErbB3's weak kinase activity means it needs to form heterodimers in order to produce effective signaling (11). Due to always being in an open conformation and therefore always being available for dimerization, ErbB2 is the preferred dimerization partner of all the other ErbBs and heterodimers containing ErbB2 provide a stronger cellular response in comparison to homodimers or other combinations of ErbBs (12). Which one of the numerous signaling pathways that are activated by ErbBs depends on which of the possible ligands (Table 1) are involved

and the combination of ErbBs in the dimer that is activated (Figure 3). Common to all the pathways is that they regulate cellular processes that typically have abnormal activity in cancers, such as apoptosis, migration, growth, adhesion, and differentiation.

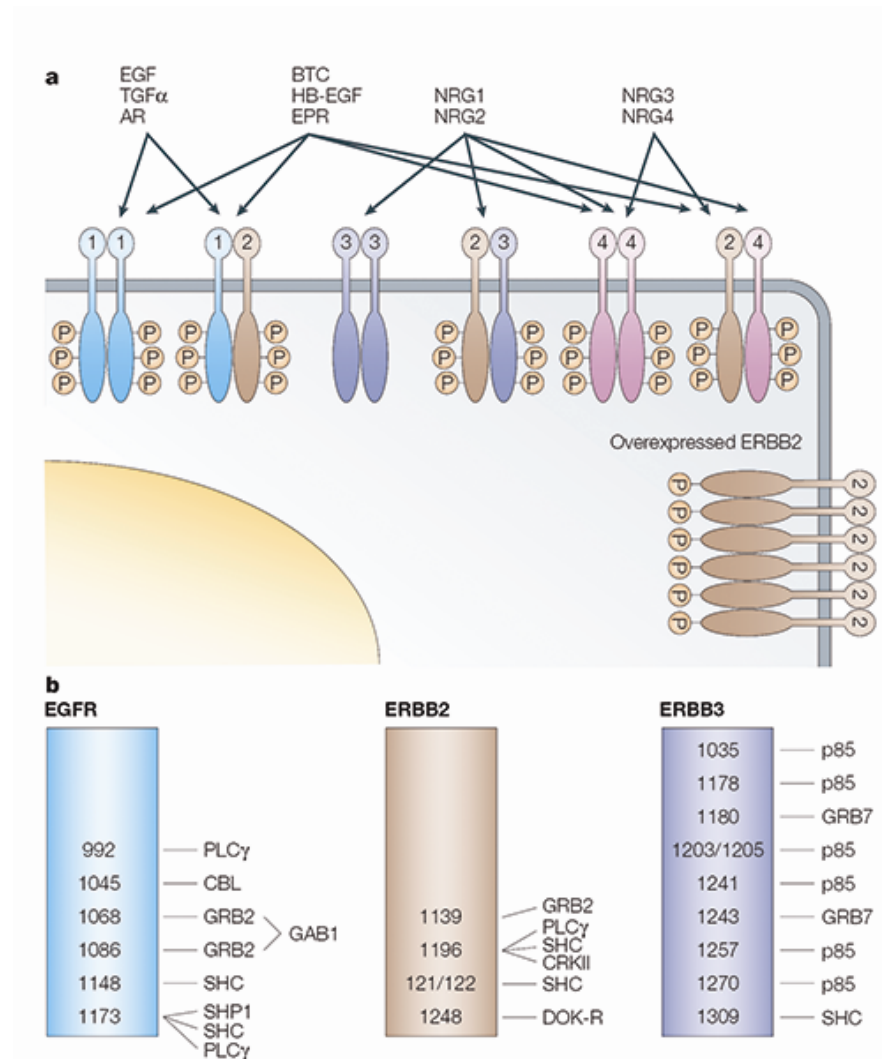


Figure 3: ErbB dimers and their ligands. (a) The possible ways in which ErbBs can dimerize with each other and which ligands activate them. Note how ErbB2, despite having no known ligand, can cluster together and create a signal when overexpressed. (b) Examples of some ErbB phosphotyrosine docking sites and the proteins which bind to them. The figure is taken from (10). The figure legend is modified.

Receptor	Remarks	Ligands
EGFR		EGF Transforming growth factor α (TGF α) Heparin-binding EGF (HB-EGF) β -Cellulin Amphiregulin Epiregulin Epigen
ErbB2	Constitutively exposed dimerization arm	
ErbB3	Kinase impaired	Heregulin-1/Neuregulin-1 Heregulin-2/Neuregulin-2
ErbB4		Heregulin-1/Neuregulin-1 Heregulin-2/Neuregulin-2 Heregulin-3/Neuregulin-3 Heregulin-4/Neuregulin-4 HB-EGF β -Cellulin Epiregulin

Table 1: ErbBs, their specialties, and their ligands (adapted from 13)

1.3.2 Endocytosis and degradation of ErbBs

1.3.2.1 EGFR

Binding of ligand to EGFR causes it to localize in clathrin-coated pits and get internalized into endocytic vesicles, causing downregulation of the receptor from the plasma membrane. Ligand-induced dimerization and the resulting phosphorylation of the receptor are necessary for coated pit recruitment (14). The phosphorylated EGFR recruits Grb2, which binds to phosphorylated tyrosine (pY) 1068 and 1086 with its SH2 domain (15). Grb2 also has two SH3 domains which recruit Cbl, an E3 ubiquitin ligase containing a RING finger domain. Cbl can also bind directly to EGFR pY1045. The Cbl proteins mediate ubiquitination of the receptor by recruitment of E2 ubiquitin conjugating enzymes (15). Ubiquitination is a process where the protein ubiquitin is covalently attached to a substrate protein where it has regulatory effects, such as serving as a signal for degradation or protein sorting, or causing activation/inactivation. Cbl with a functioning RING finger domain appears to be necessary for internalization of EGFR, suggesting that ubiquitination of the receptor

itself or other proteins associated with the receptor is required during the late phase of vesicle formation (16). It has been shown that ubiquitination of EGFR is sufficient, but not necessarily needed, for internalization (17).

Clathrin-coated vesicles containing EGFR shed their coat quickly and fuse with early endosomes (18), highly dynamic compartments that rapidly recycle its contents back to the plasma membrane, or fuse with each other to form mildly acidic sorting endosomes. At this pH, the EGF-EGFR complexes remain dimerized, phosphorylated, and ubiquitinated. This allows them to retain their tyrosine kinase activity and thus continue signaling after leaving the plasma membrane (19). Depending on the bound ligand, EGFR can be recycled back to the plasma membrane. In the case of TGF α -EGFR complexes, for example, the mild acidity of early endosomes causes the complex to dissociate, breaking up the EGFR dimer into individual proteins.

Unoccupied EGFRs are more efficiently recycled back to the cell surface (14). Intact EGFR dimers stay with the endosomes as they mature from early endosomes into late endosomes or multivesicular bodies (MVBs). During the maturation process, EGFR can be recycled back to the plasma membrane via two distinct pathways that are temperature dependent (20). Receptors that are not recycled end up inside intraluminal vesicles of MVBs (21) and are destined for lysosomal degradation.

Lysosomal targeting requires that the receptor is ubiquitinated (22). Responsible for packing EGFR into intraluminal vesicles are the ESCRT-0, -I, -II, and -III complexes which are found on the membranes of MVBs. Components of the ESCRT-0 complex, mainly Hrs, contain ubiquitin-binding domains (UBDs) which are thought to interact with ubiquitinated EGFR. Hrs also recruits clathrin to the endosomes that form a bilayered coat which is also involved in protein sorting towards lysosomes (23).

Exactly how ubiquitinated cargo ends up in vesicles inside MVBs is unclear, but since all the ESCRT complexes have lipid binding domains and UBDs, except ESCRT-III which does not have a UBD, and they facilitate the recruitment of each other in sequence (ESCRT-0 recruits ESCRT-I, ESCRT-I recruits ESCRT-II and -III), it is thought that the cargo is handed off from ESCRT-0 on to ESCRT-I and then onwards to ESCRT-II and -III (24). ESCRT-III forms a lattice-like structure that promotes invagination of the endosomal membrane which helps the budding of luminal vesicles of MVBs (25). It has also been suggested that EGFR contains di-leucine sorting signals which mediate lysosomal sorting independently of ubiquitination (26). MVBs

eventually fuse with lysosomes and its contents, EGFR included, are degraded by lysosomal proteases.

1.3.2.2 *ErbB2*

ErbB2 is resistant to down-regulation. Studies show that its localization, apart from newly synthesized ErbB2 in the ER/Golgi, is restricted to cellular protrusions on the plasma membrane, even in cancer cells where ErbB2 is overexpressed and therefore exist in constitutively active homodimers (27). Although ErbB2 has no known ligands, this does not in itself mean that it can't be internalized. However, heterodimerization between ErbB2 and other ErbBs, such as EGFR, that are otherwise internalized when ligand is bound, causes those receptors to also resist down-regulation, indicating that it is ErbB2 that confers this resistance (28, 29). The reason(s) for this is still unclear, but several possibilities have been proposed. It may be due to lacking an internalization signal or because of its association with Cdc37-Hsp90, a common chaperone complex involved in regulating the activity of many kinase proteins. When Hsp90 is inhibited by small molecules that block ATP binding, such as Geldanamycin (GA) or the GA analogue 17-AAG (30), ErbB2 is internalized. Incubation with GA causes Hsp90 to dissociate from ErbB2, and Hsp70 is recruited along with the ubiquitin ligases CHIP and Cullin-5. This causes ubiquitination, internalization, and degradation of ErbB2 (31, 32). It has been shown that ubiquitination of ErbB2 is sufficient for endocytosis and degradation (33). The down-regulation resistance could also be due to ErbB2's interaction with raft-associated flotillins, either directly or via Hsp90, as depletion of flotillin-1 and -2 leads to internalization and degradation of ErbB2 (34), or it could be because ErbB2 inhibits formation of clathrin-coated pits (35). Finally, it could be that ErbB2 is not internalization inhibited, but rather is recycled very efficiently back to the plasma membrane (36). 17-AAG induced internalization of ErbB2 seems to be mainly through CME, followed by localizing to early endosomes and then sorting to intraluminal vesicles of MVBs (37), however, other pathways could be involved as well (38). Caspase- or proteasome-mediated cleavage of the receptor's C-terminus induces internalization (39), maybe because it exposes an internalization signal, but other studies have shown that full length ErbB2 is endocytosed upon inhibition of Hsp90, so cleavage is probably not necessary (30). Internalization can also be induced

using antibodies (see section 1.5). While the ESCRT complexes are involved in sorting of EGFR to intraluminal vesicles, little is known about their involvement in sorting of ErbB2. What is known is that ErbB2 sorting to MVBs relies on proteasomal activity, as inhibiting this causes ErbB2 to accumulate in the limiting membrane of early endosomes (30, 40), but the exact reason for this remains unknown.

1.3.2.3 ErbB3 and ErbB4

Very little is known about the endocytosis and degradation of ErbB3 and ErbB4. They were originally thought to be endocytosis impaired due to lack of a tyrosine based internalization signal (41), however ErbB4 internalization has later been shown to play an important part in neuronal development (42) and a study by our group shows that ErbB3 is internalized in the absence of added ligand, independent of its phosphorylation state, in a clathrin-dependent manner (11). Both ErbB3 and ErbB4 can be ubiquitinated by the ubiquitin ligase Nedd4 (43, 44), or Nrdp1 and Itch, respectively. This ubiquitination targets the receptors for degradation which depends on proteasomal activity and lysosomal function, as degradation is halted if these are inhibited (45-47).

1.4 ErbBs in cancer

Components of the ErbB signaling network often display abnormal activity in cancer cells because of its potent effects on cell proliferation. Many types of oncogenic viruses also take advantage of it. There are several reasons why hyperactive ErbB signaling is observed in cancer cells, such as overproduction of ligands, gene amplification leading to overexpression of receptors, or mutations causing constitutively active receptors. Knowing in what way the ErbB signaling of a particular tumor has become overactive is very useful for determining patient survival chance and what sort of treatment they should receive (12).

Inappropriate release of tumor-inducing ligands can occur both paracrinely from the stroma, or autocrinely from the tumor itself. Ligands that activate EGFR are produced in a membrane-bound inactive state and only have an effect when they are released proteolytically. Inhibiting this release causes reduction in growth and migration of EGF-responsive tumor cell lines (48). TGF α is the best characterized ligand in

relation to human cancers and is correlated with poor prognosis when co-expressed with EGFR, particularly in lung, ovary and colon tumors (49).

Overexpression and/or structural changes of EGFR are both frequently found in human cancers. Studies show that the presence of a ligand is required for the overexpression of the normal receptor to be tumorigenic. Gene amplification is found together with rearrangements that yield a constitutively active receptor in a significant proportion of tumors (50).

ErbB2 is amplified and overexpressed in several human tumors, including a large percentage of breast cancers (51). The overexpression of ErbB2 facilitates the formation of ErbB2 heterodimers and the spontaneous formation of ErbB2 homodimers, the latter of which doesn't happen under normal conditions. This leads to excessive ErbB2-mediated signaling that promotes cell proliferation (12).

ErbB3's low kinase activity means that it can't cause much harm by itself. However, it can be a heterodimerization partner with other ErbBs and ErbB2 preferentially binds to it. The ErbB2-ErbB3 dimer seems to create particularly active signaling, and is important for ErbB2-mediated signaling in tumors where ErbB2 is overexpressed (52).

ErbB4 has not been studied as much as the other ErbBs in relation to its role in cancer development. ErbB4 has been proposed to be both oncogenic and tumor suppressive and the receptor has been found to be both up- and downregulated in different cancer types. The reason for these seemingly contradictory roles could be that ErbB4 has several splice variants, which have different activities (53).

Since ErbB proteins often are associated with cancer, they are attractive therapeutic targets for the development of anti-cancer drugs, including therapeutic antibodies.

1.5 Therapeutic antibodies

The use of monoclonal antibodies (mAbs) has become one of the most important and successful strategies in cancer treatment. Their targets are epitopes on the cell surface that are overexpressed, mutated or selectively expressed in cancer cells. In this way they specifically affect malignant cells while minimizing the effect on normal, healthy cells. Therapeutic mAbs can function in several ways; they can alter the function of receptors, recruit components of the immune system, or they can be conjugated to

drugs where they serve as a guidance system for specific delivery of the drug to the appropriate target cell (reviewed in 54).

1.5.1 Antibodies targeting ErbB2

Trastuzumab is a humanized mAb derived from the mouse antibody 4D5 which was shown to bind to ErbB2 and have anti-tumor activity (55). Humanization of an antibody means taking an antibody derived from some other animal and replacing most of its structure with the human equivalent, leaving only the antigen-binding variable region intact. This is done to reduce the likelihood of the human immune system reacting to the antibody, which could reduce its effectiveness. Trastuzumab binds to domain IV of ErbB2 and suppresses its signaling activity but does not lead to downregulation (56). In addition, it causes antibody-dependent cell-mediated cytotoxicity (ADCC) (57).

Pertuzumab is a humanized mAb that binds to domain II of ErbB2 (58). It inhibits dimerization by sterically blocking other ErbBs from pairing with ErbB2 (59).

Trastuzumab and pertuzumab are currently approved for clinical use and are used in conjunction with chemotherapy. Using them in combination has been shown to have a synergistic effect on the inhibition of survival of breast cancer cells, suggesting that a combination of two therapeutic antibodies to ErbB2 is more effective than treatment with individual antibodies (60-62).

The reason for this increase in effectiveness could be that a combination of two non-competing antibodies probably causes cross-linking and clustering of ErbB2-antibody complexes. This has been shown to induce efficient downregulation of ErbB2 (63, 64).

2 Project description

This project has been part of our research group's work on the characterization of two new therapeutic α -ErbB2 monoclonal antibodies developed by Symphogen A/S (Copenhagen, Denmark). The goals of the present study have been the following:

- Investigate whether the Symphogen antibodies induce internalization of ErbB2.
- Investigate whether the endocytic pathway ErbB2 is internalized by after treatment with the Symphogen antibodies is dynamin-dependent.
- Investigate the rate and efficiency of degradation of ErbB2 induced by the Symphogen antibodies.
- Evaluate the effects the Symphogen antibodies have on phosphorylation and ubiquitination of ErbB2.

Throughout the experiments conducted, particular focus has been placed on comparing the activities of the Symphogen antibodies to that of the commercially available α -ErbB2 antibodies trastuzumab and pertuzumab.

3 Methods and materials

3.1 Materials

All reagents were from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise noted. The suppliers of antibodies used are listed in Table 2.

3.1.1 Cell lines

NCI-N87 cells, a gastric carcinoma cell line that overexpresses ErbB2 (65), were given to us by Symphogen A/S.

Porcine Aortic Endothelial (PAE) cells were from Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). PAE do not produce endogenous ErbBs. The PAE cells used in this study had been stably transfected to express ErbB2 (PAE.ErbB2) (28).

3.1.2 Plasmids

pcDNA3.1-Dynamin1-K44A-HA was a gift from Sandra L. Schmid (Department of Cell Biology, UT Southwestern Medical Center, Dallas, TX, USA). The other plasmids used in this thesis, pRK5-myc-TULA and pDEST53-GFP-TULA, were made in this lab (66).

3.1.3 siRNA

For knockdown of dynamin in NCI-N87 cells a single siRNA sequence, 5'-GACAUGAUCCUGCAGUUCA-3' (67), was used.

Control cells were transfected with ON-TARGETplus Non-targeting siRNA #3 (GE Healthcare, Buckinghamshire, UK).

3.1.4 Antibodies

The therapeutic antibodies investigated in this study were provided to us by Symphogen A/S. They are monoclonal humanized antibodies that bind to domain III and IV of ErbB2 and therefore do not compete with each other for binding. They were dissolved in citrate buffer (10mM citrate, 150mM NaCl, 0.05% (v/v) Tween20, pH 6). The two antibodies will in this thesis be referred to simply as mAb1 and mAb2.

Trastuzumab, dissolved in water, was also provided to us by Symphogen A/S. Pertuzumab dissolved in L-Histidine buffer (10mM L-Histidine, 240mM sucrose, 0.02% (v/v) Tween20, pH 6) was from Roche Diagnostics GmbH (Mannheim, Germany).

Table 1 provides an overview of the antibodies used and the corresponding method:

Antigen/Species	Conjugate	Host	Dilution	Source	Method
ErbB2 (extracellular)		Mouse	1:200	Invitrogen	ICC
ErbB2 (extracellular)		Mouse	1:1000	BD	WB
ErbB2 (intracellular)		Rabbit	1:100	Invitrogen	ICC
ErbB2		Rabbit		CST	IP
ErbB2 (pY1112)		Mouse	1:200	Santa Cruz	WB
ErbB2 (pY1221)		Rabbit	1:1000	CST	WB
ErbB2 (pY1248)		Rabbit	1:1000	CST	WB
EEA1		Goat	1:500	Santa Cruz	ICC
Dynamin		Rabbit	1:200	Santa Cruz	WB
HA		Rabbit	1:1000	Abcam	ICC/WB
Myc		Mouse	1:20	H. Stenmark	ICC
Myc		Rabbit	1:5000	Abcam	ICC
Tubulin		Rabbit	1:20000	Abcam	WB
Ubiquitin		Mouse	1:500	Santa Cruz	WB
IgG/Mouse	Alexa488	Donkey	1:400	Jackson	ICC
IgG/Rabbit	RRX	Donkey	1:400	Jackson	ICC
IgG/Goat	Alexa647	Donkey	1:300	Invitrogen	ICC
IgG/Mouse	HRP	Donkey	1:20000	Jackson	WB
IgG/Rabbit	HRP	Donkey	1:20000	Jackson	WB
IgG/Human	RRX	Donkey	1:50	Jackson	ICC
IgG/Mouse	RRX	Donkey	1:400	Jackson	ICC
IgG/Rabbit	DyLight488	Donkey	1:400	Jackson	ICC
IgG/Mouse	DyLight649	Donkey	1:500	Jackson	ICC

Table 2: Antibodies used in this thesis. ICC: Immunocytochemistry, WB: Western blotting, IP: Immunoprecipitation

3.2 Methods

3.2.1 Cell culture and treatment

The NCI-N87 and PAE.ErbB2 cell lines were maintained in RPMI 1640 with L-Glutamine (Lonza, MD, USA) and Ham's F-12 with L-Glutamine (Lonza, MD, USA), respectively. The media was supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.5 x penicillin-streptomycin mixture (Lonza, MD, USA). The media used for PAE.132 cells also included 30 µg/mL of the selective antibiotic Zeocin (Life Technologies, CA, USA). The cells were incubated at 37°C in a humidified 5% CO₂, 95% air incubator. Cells were brought into suspension using 0.02% (wt/v) trypsin in 0.05% (wt/v) Ethylenediaminetetraacetic acid (EDTA) (Lonza, MD, USA).

Cells were treated with antibodies in a 25 µg/ml total concentration. Cells were treated with either only mAb1, only mAb2, only Trastuzumab, only Pertuzumab, or a combination of both mAb1 and mAb2, or a combination of Trastuzumab and Pertuzumab, in a 1:1 ratio. As a positive control, cells were treated with 3 µM of 17-AAG. All incubations were done at 37°C. For the immunoprecipitation experiment, where incubation time was short, minimum essential media (MEM) without bicarbonate (Life Technologies, CA, USA) with 0.1% (wt/v) bovine serum albumin (BSA) was used.

3.2.2 Transfection

Transfection is the process of introducing nucleic acids into a eukaryotic cell. The method used here is lipofection, which complexes the negatively charged nucleic acids with positively charged lipids. These liposomes can fuse with the negatively charged plasma membrane and release its contents into the cell's cytosol.

3.2.2.1 Transfection with siRNA

Small interfering RNA (siRNA) transfection is used to transiently knock down the expression of specific genes by causing its associated mRNA to be degraded and thus not to be translated into a functional protein.

Cells were seeded on 24-well plates in antibiotics-free medium to a concentration of 1x10⁵ cells/ml 24 hours prior to transfection. An siRNA solution was made by taking 1.5 µl/well of a 20 µM siRNA solution and mixing it with 50 µl/well of Opti-MEM

(Life Technologies, CA, USA). A Lipofectamine RNAiMAX mix was made by mixing 1.5µl/well of Lipofectamine RNAiMAX Reagent (Life Technologies, CA, USA) with 50µl/well of Opti-MEM. This mix was added to the siRNA solution and left to incubate at room temperature (RT) for 20 minutes. After this, 100µl/well of transfection solution was added to the 500µl cell growth media in the wells making a final siRNA concentration of 50nM. The cells were then left to incubate at 37°C for 72 hours prior to experiments.

3.2.2.2 Transfection with plasmid

Transfection with a plasmid is used to introduce new genes into a cell, making it express that gene.

Cells were seeded on 24-well plates in antibiotics-free medium to a concentration of 8×10^4 cells/ml 24 hours prior to transfection. A mix of 50µl/well of Opti-MEM and 0.5µg/well of plasmid was left to incubate at RT for 5 minutes, after which 1µl/well of Lipofectamine LTX Reagent (Life Technologies, CA, USA) was added. The transfection solution was then left to incubate at RT for 30 minutes before 50µl was added to the 500µl cell growth media in each well. The cells were left to incubate at 37°C for 24 hours prior to experiments.

3.2.3 Western blot

The western blot technique is used to separate out and detect specific proteins from a cell lysate or other solution containing different proteins mixed together. It is useful for quantifying the amount of a certain protein in a sample, its expression level, or for determining protein modification, such as phosphorylation. This is accomplished by incubating the proteins with sodium dodecyl sulfate (SDS), which denatures them and applies a negative charge, allowing them to be separated according to molecular weight by polyacrylamide gel electrophoresis (PAGE). The proteins can then be transferred from the gel to a nitrocellulose membrane that can be probed for proteins of interest using specific primary antibodies coupled with a signal-inducing secondary antibody.

After treatment, cells were washed three times with RT phosphate-buffered saline (PBS) (137mM NaCl (Prolabo, PA, USA), 2.7mM KCl, 1mM Na₂HPO₄, 2mM NaH₂PO₄, pH 7.4) and then quickly lysed with SDS-lysis buffer (10mM Tris, pH 6.8, 5mM EDTA, 50mM NaF, 30mM sodium pyrophosphate, 2% (wt/v) SDS (Applichem GmbH, Darmstadt, Germany), 1mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Fluka Chemie AG, Buchs, Switzerland) was added to the lysis buffer prior to each experiment, phosphatase inhibitor cocktail was also added for experiments involving phosphorylation) at RT. The resulting solutions were transferred to QIAshredder columns (Qiagen, Hilden, Germany) and centrifuged at 4°C, at 20000 x g for 2 minutes to remove debris and reduce viscosity. Sample buffer (4% (v/v) glycerol, 4% (v/v) β-mercaptoethanol and 0.005% bromophenol blue) was added to the lysates before incubation at 95°C for 10 minutes followed by a quick centrifugation.

The samples were subjected to SDS-PAGE, using precast gels (4% stacking gel, 10% separating gel) (Bio-Rad Laboratories, CA, USA), for 15-20 minutes at 300V.

Proteins were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, CA, USA) for 12 minutes at 25V using a Trans-Blot Turbo System (Bio-Rad Laboratories, CA, USA). Membranes were then washed once in Tris-buffered saline with Tween20 (TBST) (10mM Tris, pH7.6, 137mM NaCl, 0.1% (v/v) Tween 20) and blocked for non-specific interactions using 5% (wt/v) Blotting Grade Blocker (Bio-Rad Laboratories, CA, USA) in TBST for at least 20 minutes at RT, followed by incubation with primary antibody diluted in 1% (wt/v) Blotting Grade Blocker in TBST for 1-2 hours at RT or overnight at 4°C. After this the membrane was washed for 5-10 minutes in TBST three times and then blocked again as described earlier. It was then incubated with horseradish peroxidase (HRP) conjugated secondary antibody in the same manner as the primary antibody followed, finally, by washing with TBST for 5-10 minutes 3-4 times.

Antibody-labeled proteins on the membrane were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, MA, USA), which upon exposure to HRP luminesce. The light from this reaction was captured by a ChemiDoc MP Imaging System (Bio-Rad Laboratories, CA, USA) and analyzed using Image Lab software.

3.2.4 Immunocytochemistry and confocal microscopy

Immunocytochemistry is used to find the cellular localization of proteins using protein-specific primary antibodies, conjugated to a fluorophore, or in combination with fluorophore-conjugated secondary antibodies. A laser with light of a specific wavelength is used to excite the fluorophore-conjugated antibody. This causes it to emit light of a different wavelength which is then detected using a confocal microscope which captures light only at the focal plane while blocking out of focus light by use of a pinhole. This increases resolution in the depth direction of the cell and thus allows a high degree of accuracy when determining the location of a protein in all three spatial dimensions.

Cells were plated on glass coverslips coated in fibronectin to aid in cell adhesion. After treatment, cells were washed once with RT PBS and then fixed with heated (37°C) Formalin Solution 10% for 10 minutes to preserve morphology and terminate all biological processes. The cells were then washed for 5 minutes with PBS three times. Quenching with 50mM NH_4Cl for 10 minutes was carried out to prevent aldehyde in the Formalin Solution from reacting with amines and proteins, which can create fluorescent products that can cause false positive and high background detection. Then they were washed quickly with PBS twice. Cells were then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 minutes to allow antibodies passage through the cell membrane so that they can bind to intracellular antigens. A quick wash in PBS followed before blocking non-specific binding sites with 1% (wt/v) BSA in PBS for 30 minutes. After that, the cells were incubated with primary antibody diluted in 1% (wt/v) BSA for 1 hour, and then washed in PBS three times for 10 minutes each. Incubation with secondary antibody was then done in the same manner as described for the primary antibody, followed by another round of three 10 minute washes with PBS.

The coverslips were finally mounted on microscope slides using Dako fluorescent mounting medium (Dako Corporation, Copenhagen, Denmark) and examined using an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan) with Olympus FluoView software. Final image editing was done in ImageJ and Adobe Photoshop CS6.

3.2.5 Immunoprecipitation

Immunoprecipitation (IP) enables the precipitation of proteins using antibodies for a particular protein coupled to a solid-phase substrate. The technique allows for the isolation of a specific protein from a sample containing thousands of different proteins. The IPs were done under denaturing conditions to prevent interaction partners from being co-precipitated.

Cells were treated for 1 hour at 37°C after which they were washed 3 times with RT PBS and then lysed using pre-warmed 1% (wt/v) SDS in PBS at 100°C and boiled for 5 minutes. After this, the lysates were transferred to pre-cooled QIAshredder columns and kept on ice briefly before being centrifuged at 20000 x g at 4°C for 2 minutes. Protein G-coupled Dynabeads (Life Technologies, CA, USA) were washed 4 times with protein G binding buffer (0.05% (v/v) Triton X-100, 0.2 M sodium phosphate, 0.2 M citric acid monohydrate, pH 5.0) and incubated with α -ErbB2 antibody at RT for 1 hour on a rotor. Afterwards, the beads were washed 4 times with 1X IP buffer (2X IP buffer (2% (v/v) Triton X-100, 0.5% (v/v) Na-deoxycholate, 1% (wt/v) BSA, 2mM EDTA, 40mM sodium fluoride (NaF) in PBS) diluted 1:1 with 1% (wt/v) SDS in PBS, protease inhibitor cocktail, phosphatase inhibitor cocktail, and N-ethylmaleimide (NEM) to inhibit deubiquitinases, was added to the IP buffer prior to each experiment) and then resuspended in 2X IP buffer and stored on ice. Cell lysate and antibody-bound protein G-coupled Dynabeads were mixed in a 1:1 ratio and incubated at 4°C for 1 hour on a rotor. The solution was then washed 4 times with 1X IP buffer followed by a final wash with 10% (v/v) PBS in ddH₂O. The antibody-protein complexes were eluted with 2X sample buffer (20mM Tris-HCl (pH 6.8), 10mM EDTA, 100mM NaF, 60mM sodium pyrophosphate, 4% (wt/v) SDS, 2% (v/v) β -mercaptoethanol, 20% (v/v) glycerol, 0.006% (wt/v) bromophenol blue) at 95°C for 10 minutes and then centrifuged briefly. The samples with immunoprecipitated protein were then subjected to SDS-PAGE and western blot as described in section 3.2.3.

4 Results

All experiments have been conducted three times. Figures show a representative result.

4.1 Treatment with Symphogen α -ErbB2 antibodies causes internalization of ErbB2

To investigate the cellular localization of ErbB2 upon treatment with the Symphogen α -ErbB2 antibodies, NCI-N87 cells were incubated with a mixture of mAb1 and mAb2 (hereafter referred to as mAb mix), trastuzumab and pertuzumab, or 17-AAG for 4 hours. The cells were then subjected to immunocytochemical labeling where they were labeled with mouse α -ErbB2 antibody, which were visualized by confocal microscopy (Figure 4). mAb1 and mAb2 were not tested by themselves because we have previously observed that they do not induce effective internalization (data not shown). 17-AAG was included as a positive control since it is known that it induces internalization of ErbB2. There was significant localization of ErbB2 to internal vesicles in all three cases. The 17-AAG was more effective than the mAb mix. The mAb mix appears to cause more internalization than the trastuzumab and pertuzumab combination, which has previously been shown to induce internalization of ErbB2 (61).

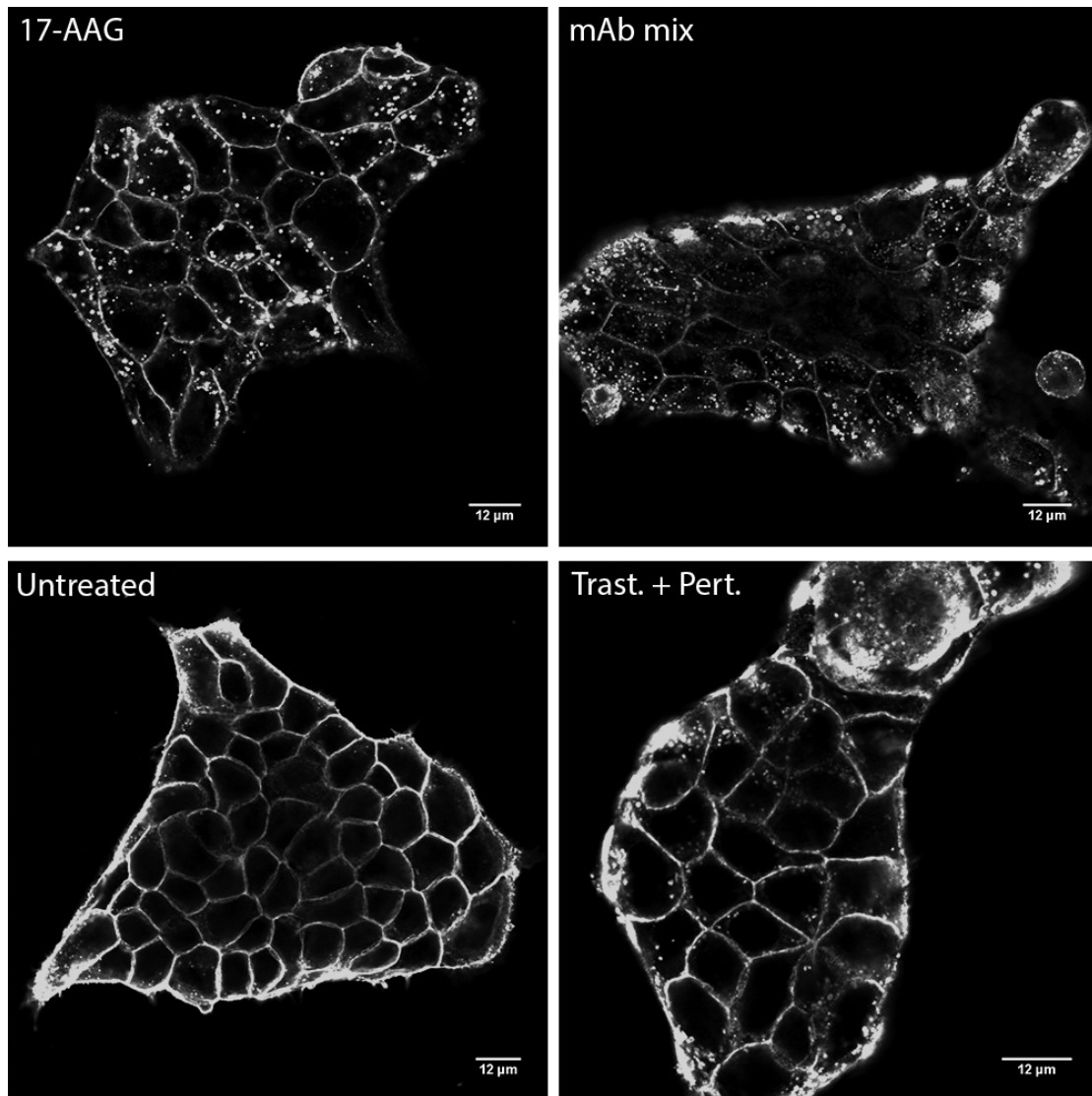


Figure 4: Treatment with Symphogen α -ErbB2 antibodies causes internalization of ErbB2. NCI-N87 cells were incubated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for 4 hours. They were then labelled with mouse α -ErbB2 antibody, followed by Alexa488 donkey α -mouse antibody. Detection of ErbB2 was done by confocal microscopy.

4.2 ErbB2 localizes to EEA1-positive endosomes upon incubation with the mAb mix

To further investigate the subcellular localization of ErbB2 after internalization, the NCI-N87 cells had also been labeled with antibody to EEA1. Analysis by confocal microscopy showed partial colocalization between ErbB2 and EEA 1-positive endosomes (Figure 5).

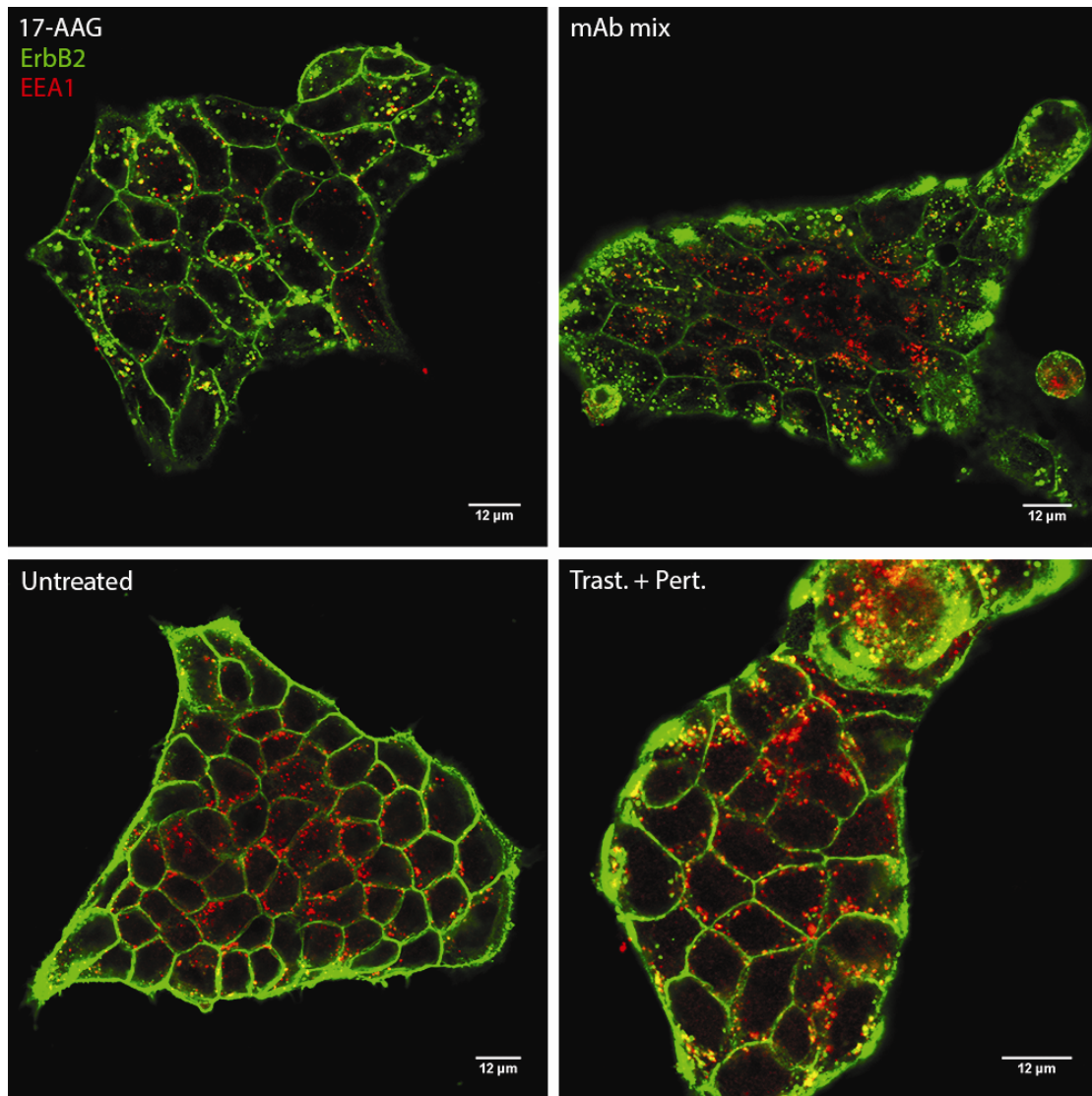


Figure 5: ErbB2 localizes to EEA1-positive endosomes. NCI-N87 cells were incubated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for 4 hours. They were then labelled with mouse α -ErbB2 antibody and goat α -EEA1 antibody, followed by Alexa488 donkey α -mouse antibody and Alexa647 donkey α -goat antibody. Detection of ErbB2 and EEA1 was done by confocal microscopy. Yellow spots correspond to colocalization of ErbB2 and EEA1.

4.3 ErbB2 is degraded upon incubation with the mAb mix

To find out whether ErbB2 is degraded upon incubation with the mAb mix, NCI-N87 cells were incubated under a variety of conditions (Figure 6) for 24 hours and then lysed, subjected to SDS-PAGE and analyzed by western blotting using a mouse α -ErbB2 antibody. The antibodies were tested both individually and in combination. 17-AAG was used as positive control for degradation. As negative controls, an antibody that doesn't bind to the cells (Neg. Ctr. Ab) and citrate buffer, which is what the

mAbs are dissolved in, were used. The Symphogen α -ErbB2 antibodies caused more degradation than trastuzumab and partuzumab. In both cases a combination of the antibodies was more effective than the individual antibodies on their own.

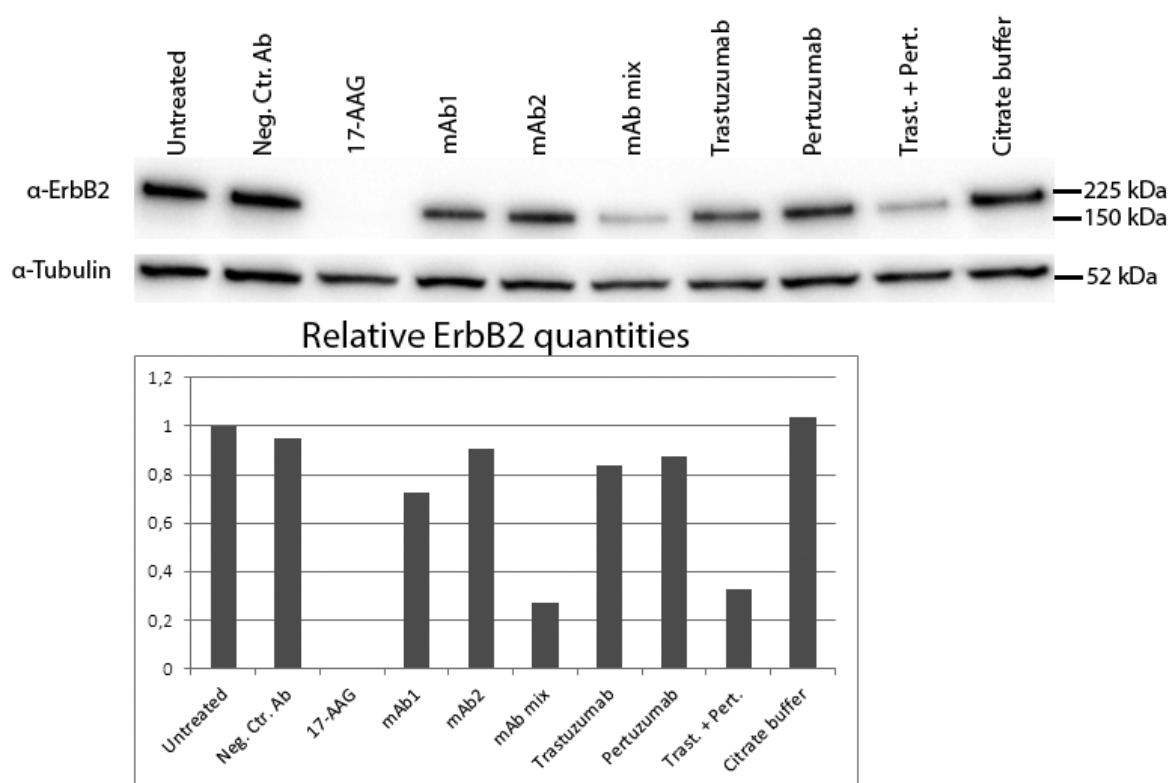


Figure 6: ErbB2 is degraded upon incubation with the mAb mix. NCI-N87 cells were incubated under the conditions shown for 24 hours. The cells were lysed, subjected to SDS-PAGE and analysed by western blotting using a mouse α -ErbB2 antibody and an HRP-conjugated α -mouse secondary antibody. ErbB2 quantities were normalized using tubulin as a loading control.

4.4 Dynamin knockdown does not impede mAb mix-induced ErbB2 degradation

In order to investigate whether antibody-induced endocytosis of ErbB2 is dynamin-dependent, dynamin was transiently knocked down in NCI-N87 cells using siRNA prior to treatment. Non-targeting siRNA was used as control. Cells were incubated under the same conditions as described in section 4.3, with the exception of the 17-AAG which cells were only incubated with for 4 hours because the combination of transfection, dynamin knockdown and 24 hours of 17-AAG caused cell death. After treatment the cells were lysed and analyzed by western blotting using antibodies

against dynamin and the extracellular region of ErbB2. Dynamin knockdown did not appear to have an effect on ErbB2 degradation under any of the conditions. Not even the 17-AAG positive control, which has previously been shown to be dynamin-dependent, seemed to be affected (Figure 7). This prompted further investigation.

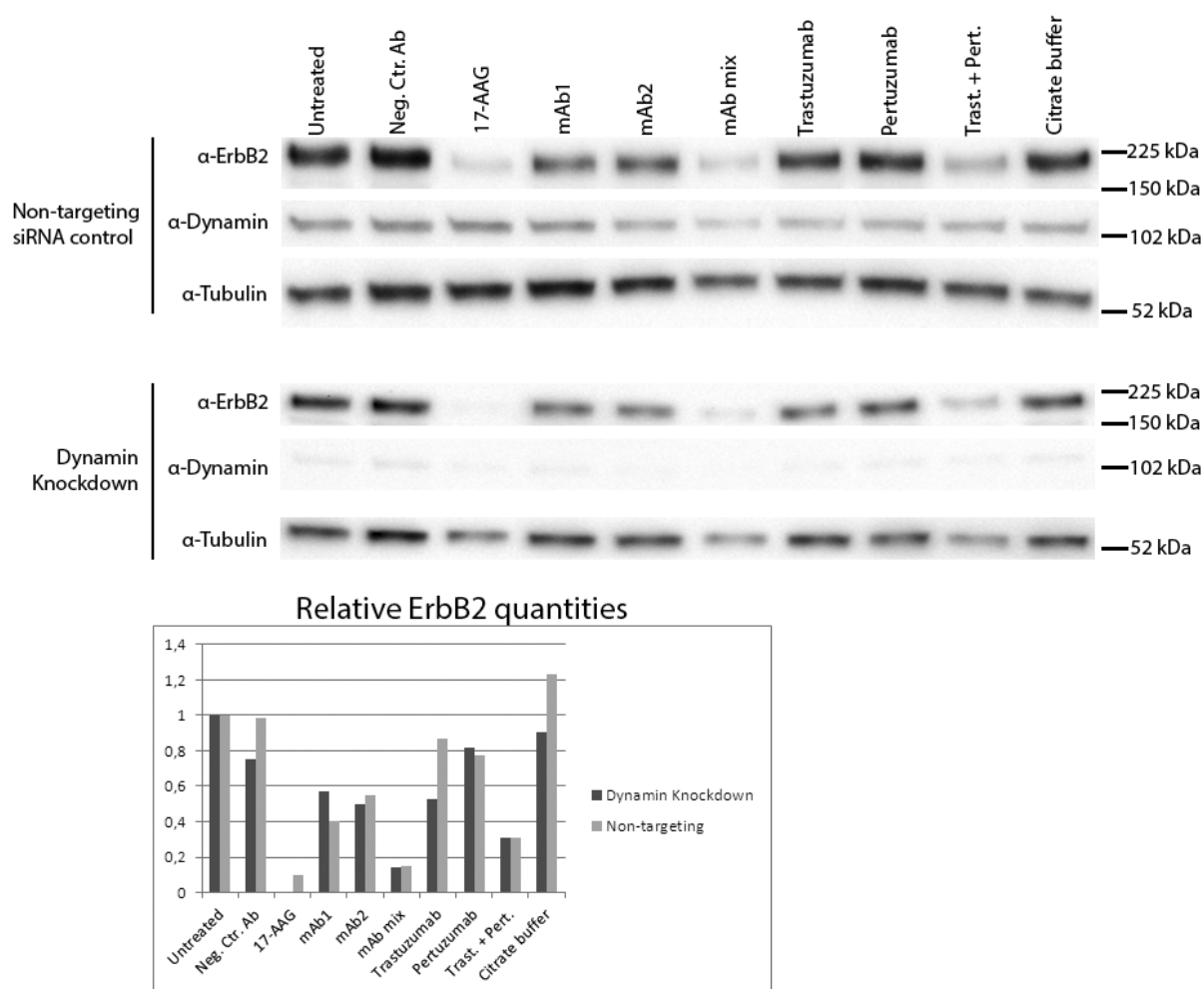


Figure 7: Dynamin knockdown does not impede mAb mix-induced ErbB2 degradation. Dynamin was knocked down in NCI-N87 cells, which were then incubated under the conditions shown for 24 hours, with the exception of 17-AAG which cells were incubated with for 4 hours. The cells were lysed, subjected to SDS-PAGE and analysed by western blotting using mouse α -ErbB2 antibody and rabbit α -dynamin antibody, followed by HRP-conjugated α -mouse and α -rabbit antibodies, respectively. The exposure time was the same for the two membranes showing dynamin amounts. ErbB2 quantities were normalized using tubulin as loading control.

Previous experiments by this group have shown that when GA-induced ErbB2 internalization is inhibited by clathrin knockdown, the internal region of the receptor is detached and degraded (30). This leaves a truncated fragment on the plasma membrane consisting of the extracellular and transmembrane regions of the receptor. With the way the membrane was cut in Figure 7, this fragment would have been too low down to have been labeled. Because of this, an additional experiment was conducted on the same lysates to look for the smaller fragment which, if present, would indicate that antibody-induced internalization of ErbB2 is dynamin-dependent. Analysis by western blotting using an antibody against the extracellular region of ErbB2, on the entire membrane this time, confirmed that there was a fragment corresponding to the truncated ErbB2 in all of the lysates, but the one that was treated with 17-AAG and had dynamin knocked down showed a much stronger band. Cells treated with Symphogen α -ErbB2 antibodies or trastuzumab and pertuzumab showed no difference from the untreated control in this regard (Figure 8).

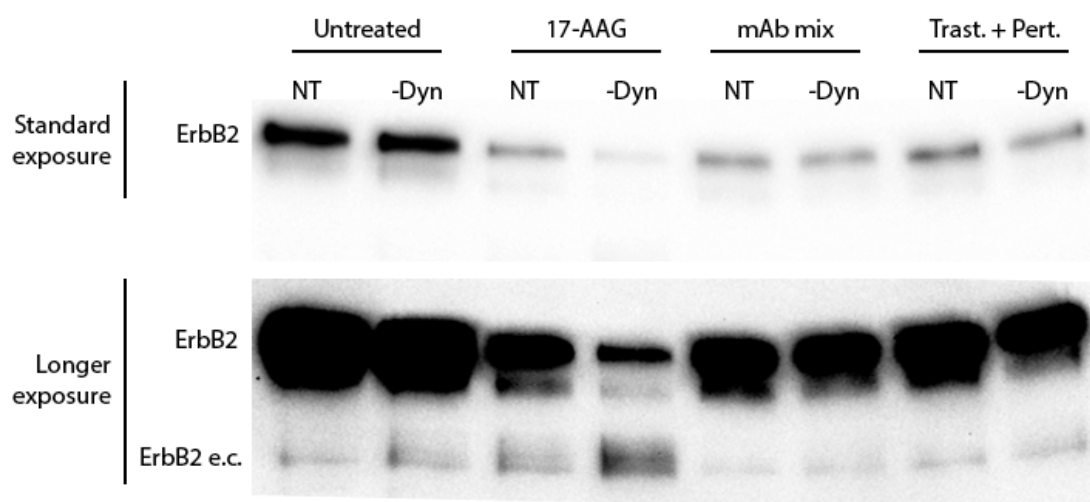


Figure 8: Treatment with 17-AAG produces a truncated ErbB2 fragment when dynamin is knocked down with DYN2 siRNA. An antibody against the extracellular region of ErbB2 was used to label both the full length protein and the truncated fragment. A secondary HRP-conjugated antibody was used to produce the signal. Long exposure time had to be used to detect the fragment, so the bands showing the full length ErbB2 protein are saturated.

4.5 Treatment with the mAb mix induces internalization and colocalization of ErbB2 with EEA1-positive endosomes in PAE.ErbB2 cells

To further investigate what happens to internalization of ErbB2 when dynamin function is impaired, transient transfection of a plasmid containing the gene for K44A dynamin, a mutated version of dynamin lacking GTPase activity, was attempted on NCI-N87 cells. These cells proved to be difficult to transfect, so PAE.ErbB2 cells were used instead. Because of this switch in cell type, the previously described experiments were repeated to ensure that the findings regarding internalization and localization of ErbB2 in NCI-N87 cells were the same in PAE.ErbB2 cells. The experiments were conducted in the same way as described for NCI-N87 cells. Results were similar and showed internalization and colocalization with EEA1-positive endosomes after antibody treatment (Figure 9, 10).

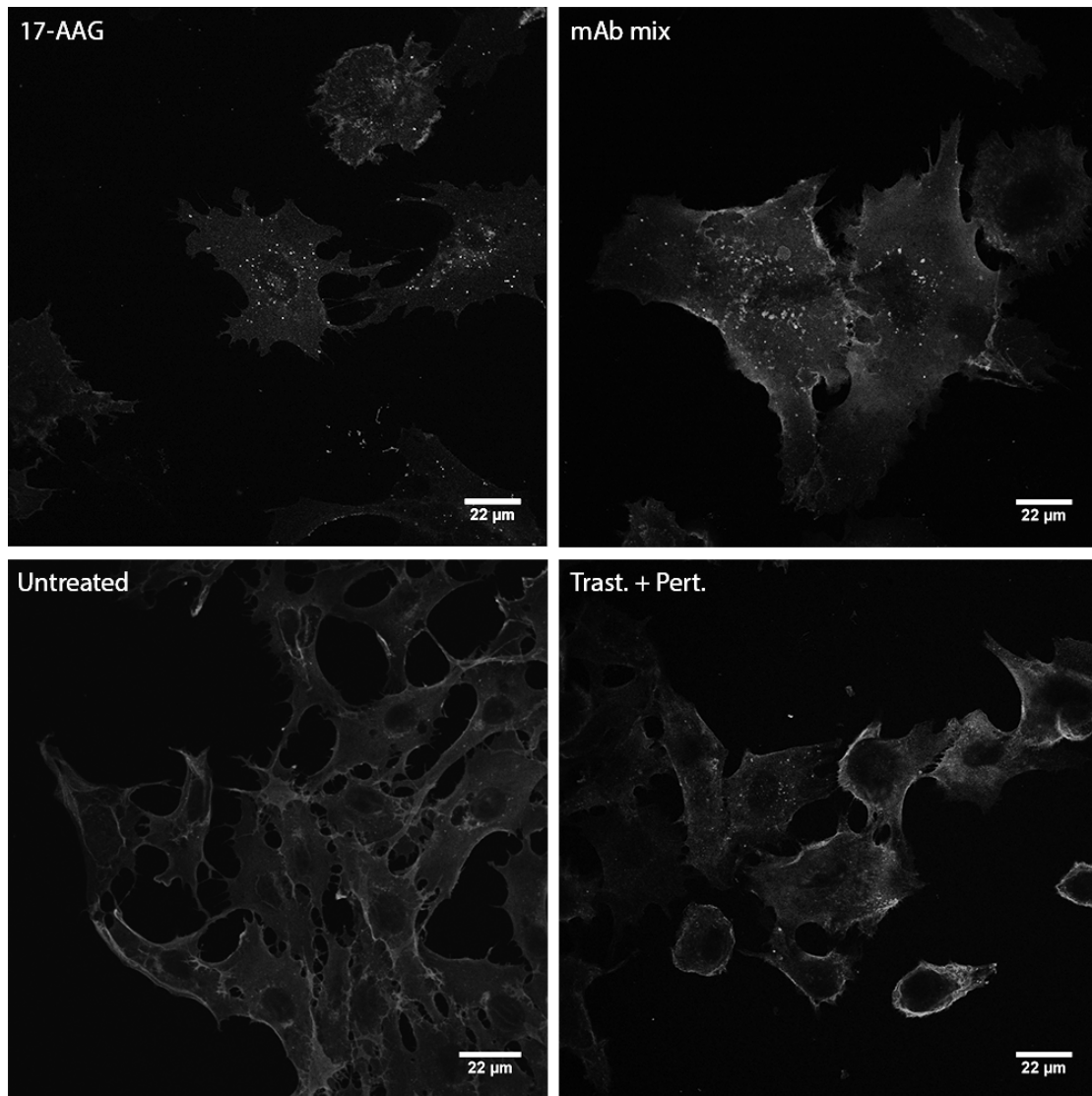


Figure 9: Treatment with the mAb mix causes internalization of ErbB2 in PAE.ErbB2 cells.

PAE.ErbB2 cells were incubated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for 4 hours. They were then labelled with mouse α -ErbB2 antibody, followed by Alexa488 donkey α -mouse antibody. Detection of ErbB2 was done by confocal microscopy.

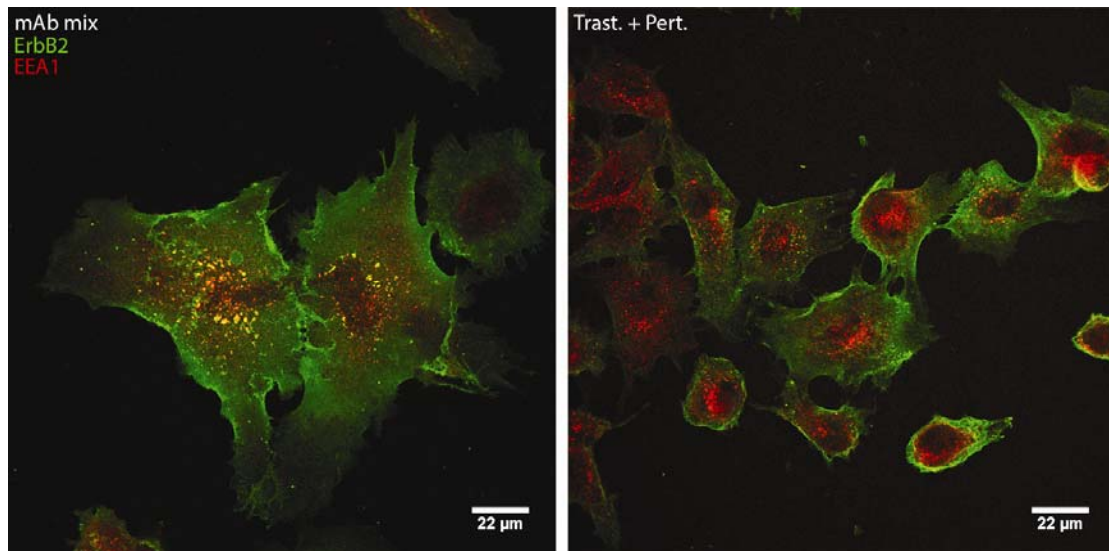


Figure 10: ErbB2 localizes to EEA1-positive endosomes upon incubation with the mAb mix.

PAE.ErbB2 cells were incubated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for 4 hours. They were then labelled with mouse α -ErbB2 antibody and goat α -EEA1 antibody, followed by Alexa488 donkey α -mouse antibody and Alexa647 donkey α -goat antibody, respectively. Detection of ErbB2 and EEA1 was done by confocal microscopy. Yellow spots correspond to colocalization of ErbB2 and EEA1.

4.6 Expression of K44A dynamin reduces ErbB2 internalization

As mentioned earlier, cells were transiently transfected with plasmid containing the K44A dynamin gene to observe whether it had an effect on antibody-induced ErbB2 internalization. K44A dynamin's lack of GTPase activity means that it should work as an inhibitor of dynamin-dependent endocytosis by acting as a competitive inhibitor of the cell's normal, functioning dynamin. The mutated dynamin also includes an HA-tag, which makes it possible to immunostain it without also staining for regular dynamin. In this way, cells that are expressing K44A dynamin can be visually distinguished from cells that do not in the microscope. Transiently transfected PAE.ErbB2 cells were incubated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for 4 hours. The cells were fixed and then labeled for ErbB2 and HA and analyzed by confocal microscopy. Internalization of ErbB2 appeared to be reduced in K44A dynamin expressing cells compared to other cells, with more ErbB2 still left on the plasma membrane. There wasn't a complete block of internalization under any of the conditions, though 17-AAG-induced internalization was more efficiently impaired than either of the antibody combinations. The amount of internalized ErbB2 seemed

inversely proportional to how strong the K44A dynamin expression was. As had been observed in other experiments, internalization induced by the Symphogen α -ErbB2 antibodies seemed more effective than trastuzumab and pertuzumab (Figure 11).

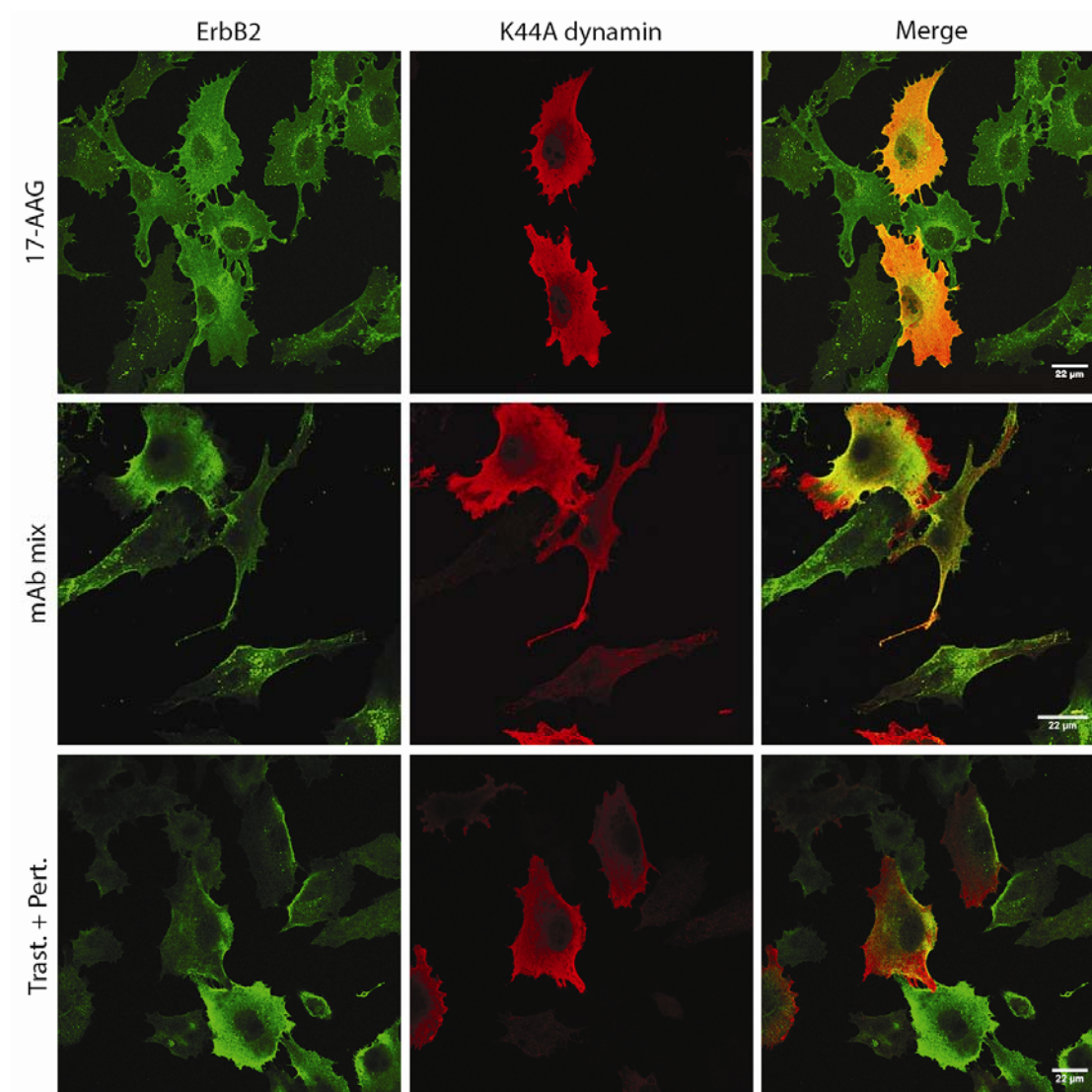


Figure 11: ErbB2 internalization is reduced in PAE.ErbB2 cells expressing K44A dynamin. The cells were incubated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for 4 hours. They were then labelled with mouse α -ErbB2 antibody and rabbit α -HA antibody, followed by Rhodamine Red-X α -mouse antibody and Alexa488 α -rabbit antibody. Detection was done by confocal microscopy.

4.7 Symphogen α -ErbB2 antibodies are internalized along with ErbB2

To find out whether the α -ErbB2 antibodies are internalized along with ErbB2, mAb mix treated PAE.ErbB2 cells were labeled with α -human IgG antibody. The α -human IgG antibody will bind to the Symphogen α -ErbB2 antibodies attached to ErbB2 and analysis by confocal microscopy showed that the Symphogen α -ErbB2 antibodies localize to EEA1-positive early endosomes (Figure 12). An untreated control was used to confirm that the α -human IgG antibody does not bind to any part of the cells themselves (data not shown).

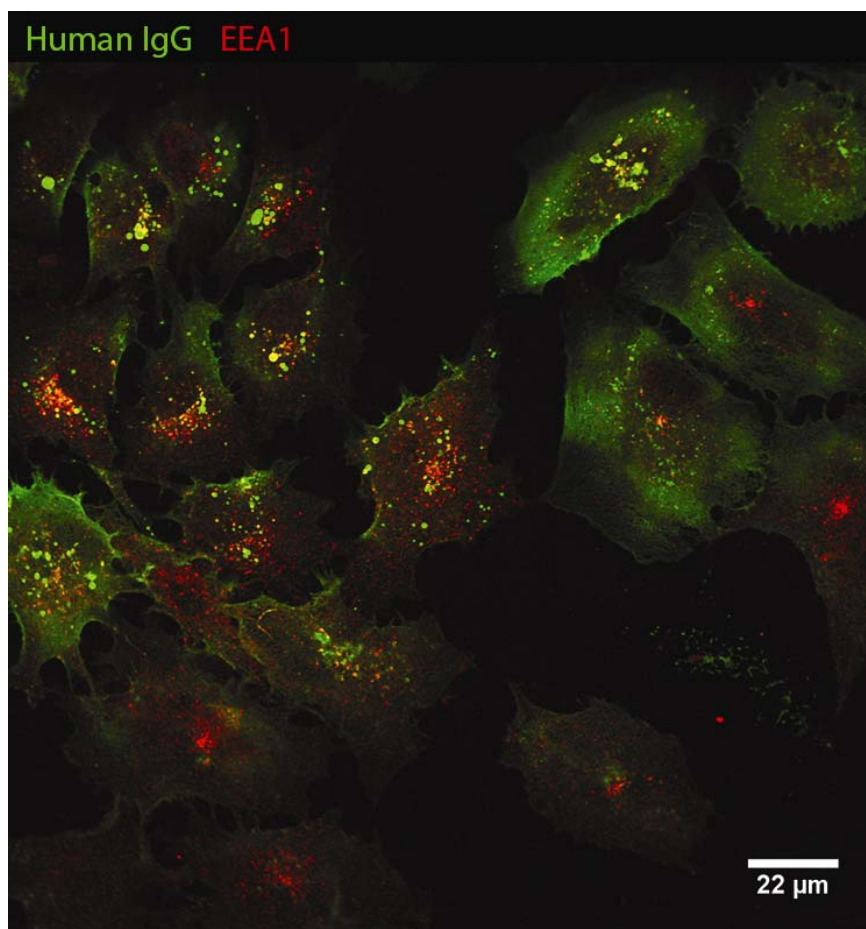


Figure 12: Symphogen α -ErbB2 antibodies are internalized along with ErbB2. PAE.ErbB2 cells were incubated with mAb mix for 4 hours. They were then labelled with Rhodamine Red-X α -human IgG antibody, and goat α -EEA1 antibody followed by Alexa647 α -goat antibody. Analysis was done by confocal microscopy. Yellow spots correspond to colocalization of Symphogen α -ErbB2 antibodies and EEA1. Some of the cells were expressing K44A dynamin, which is why some have much stronger human IgG labelling on the membrane than others.

4.8 Transient K44A dynamin expression in PAE.ErbB2 did not block ErbB2 degradation

With the finding that expression of K44A dynamin seems to affect ErbB2 antibody-induced internalization, experiments were performed to see if it had an effect on degradation. Initially the experiment was supposed to be conducted on PAE cells without ErbB2 expression, which would be co-transfected with both a plasmid containing the ErbB2 gene and one with the K44A dynamin gene. The idea was that cells would be transfected with both plasmids so that most cells expressing ErbB2 would also express K44A dynamin. In this way studies of ErbB2 would be mainly limited to K44A dynamin transfected cells and it would be possible to see if degradation was affected. However, the cells didn't survive co-transfection, so this was not possible. Instead, PAE.ErbB2 cells were transfected with K44A dynamin, to see if it had a detectable effect despite the fact that the lysate would include ErbB2 from untransfected cells. After transfection, the cells were treated mAb mix or trastuzumab and pertuzumab for 24 hours, or 17-AAG for 4 hours, and then lysed, subjected to SDS-PAGE and analyzed by western blot. The blot was labeled for ErbB2, dynamin and HA. The analysis showed that transfection of PAE.ErbB2 cells with K44A dynamin did not have a noticeable effect on antibody-induced degradation of ErbB2. While the HA-tag labeling shows that there were successful transfections, it did not produce a noticeable increase in total dynamin compared to the controls, suggesting that the expression of K44A dynamin was relatively low (Figure 13).

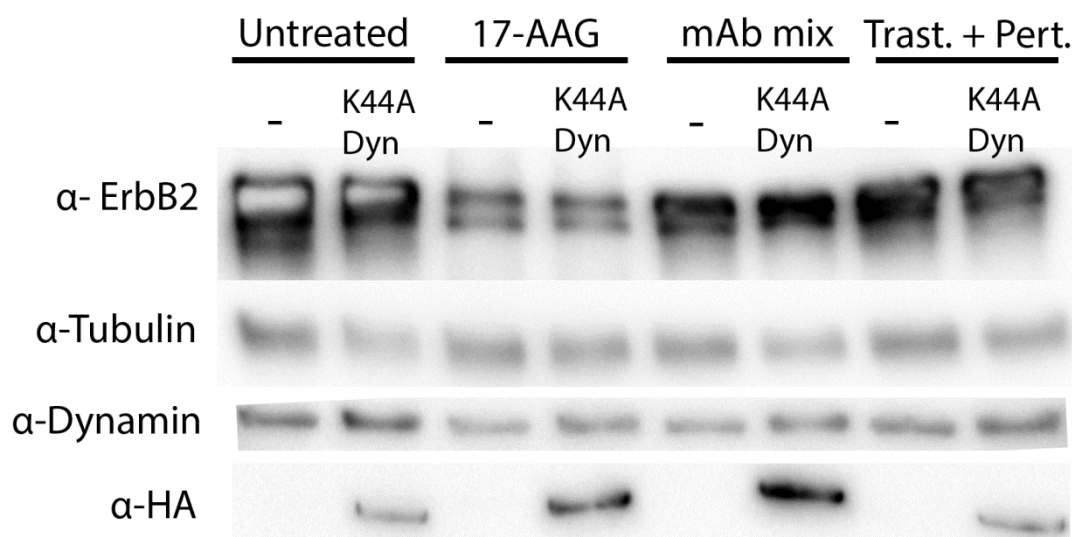


Figure 13: Transfection of PAE.ErbB2 cells with K44A dynamin does not have a noticeable effect on degradation of ErbB2. The cells were incubated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for 24 hours. They were then lysed, subjected to SDS-PAGE and analysed by western blotting using mouse α -ErbB2 antibody, rabbit α -dynamin antibody, and rabbit α -HA antibody, followed by HRP-conjugated α -mouse and α -rabbit secondary antibodies. The “ghost bands” seen for ErbB2 in the untreated cells are due to high concentrations of HRP, which uses up all the substrate and thus causes white spots in the bands.

4.9 T-cell ubiquitin ligand (TULA) reduces ErbB2 internalization and causes removal of ErbB2 intracellular region

To further examine the dynamin-dependency of the internalization of ErbB2 and find out whether the intracellular and extracellular region of ErbB2 remain intact when dynamin function is impaired, an alternative to K44A dynamin was used to inhibit dynamin-dependent endocytosis. TULA is a Cbl-interacting protein that has been shown to inhibit dynamin-dependent pathways, but not dynamin-independent pathways (66). PAE.ErbB2 cells were transfected with either of two plasmids containing the TULA-gene before incubation with mAb mix or 17-AAG for 4 hours. The TULA proteins included either a myc- or a GFP-tag for detection. Two separate experiments were conducted; in the first one, myc-TULA transfected cells were incubated with mAb mix and then labeled with antibodies against myc and either the intracellular region or the extracellular region of ErbB2. Analysis by confocal microscopy showed that in TULA-expressing cells, the extracellular region of ErbB2 remained in the plasma membrane while labeling for the intracellular region was

reduced (Figure 14). To observe this in individual cells, a follow-up experiment was conducted where GFP-TULA transfected cells were incubated with mAb mix or 17-AAG and then labeled for both the extracellular and intracellular region of ErbB2. Expression of GFP-TULA allowed detection of successfully transfected cells and analysis by confocal microscopy showed the same as the first experiment. In cells expressing TULA, the extracellular region of ErbB2 is retained on the plasma membrane while labeling for the intracellular region is reduced (Figure 15).

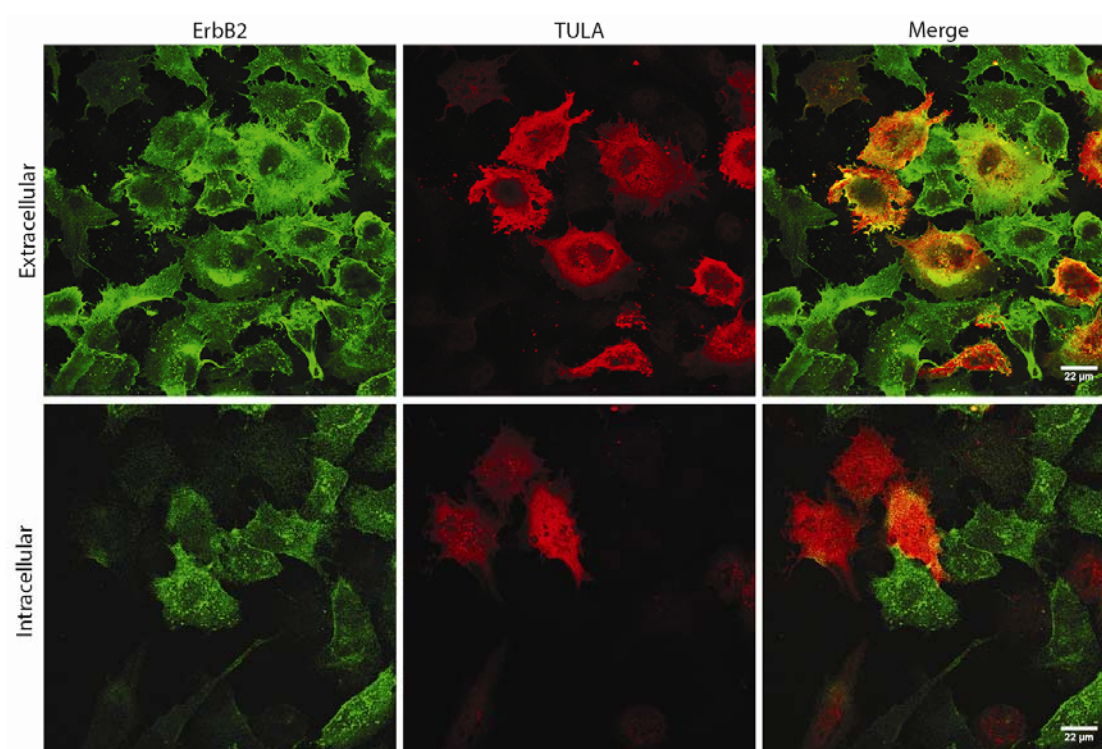


Figure 14: Inhibition of dynamin-dependent endocytosis causes retention of ErbB2 on the plasma membrane upon incubation with the mAb mix, but induces degradation of the intracellular region of ErbB2. PAE.ErbB2 cells transfected with myc-TULA were incubated with mAb mix for 4 hours. The cells were then labelled with mouse α -ErbB2 (extracellular region) antibody followed by DyLight488 α -mouse antibody, or rabbit α -ErbB2 (intracellular region) antibody followed by DyLight488 α -rabbit antibody. Detection was done by confocal microscopy.

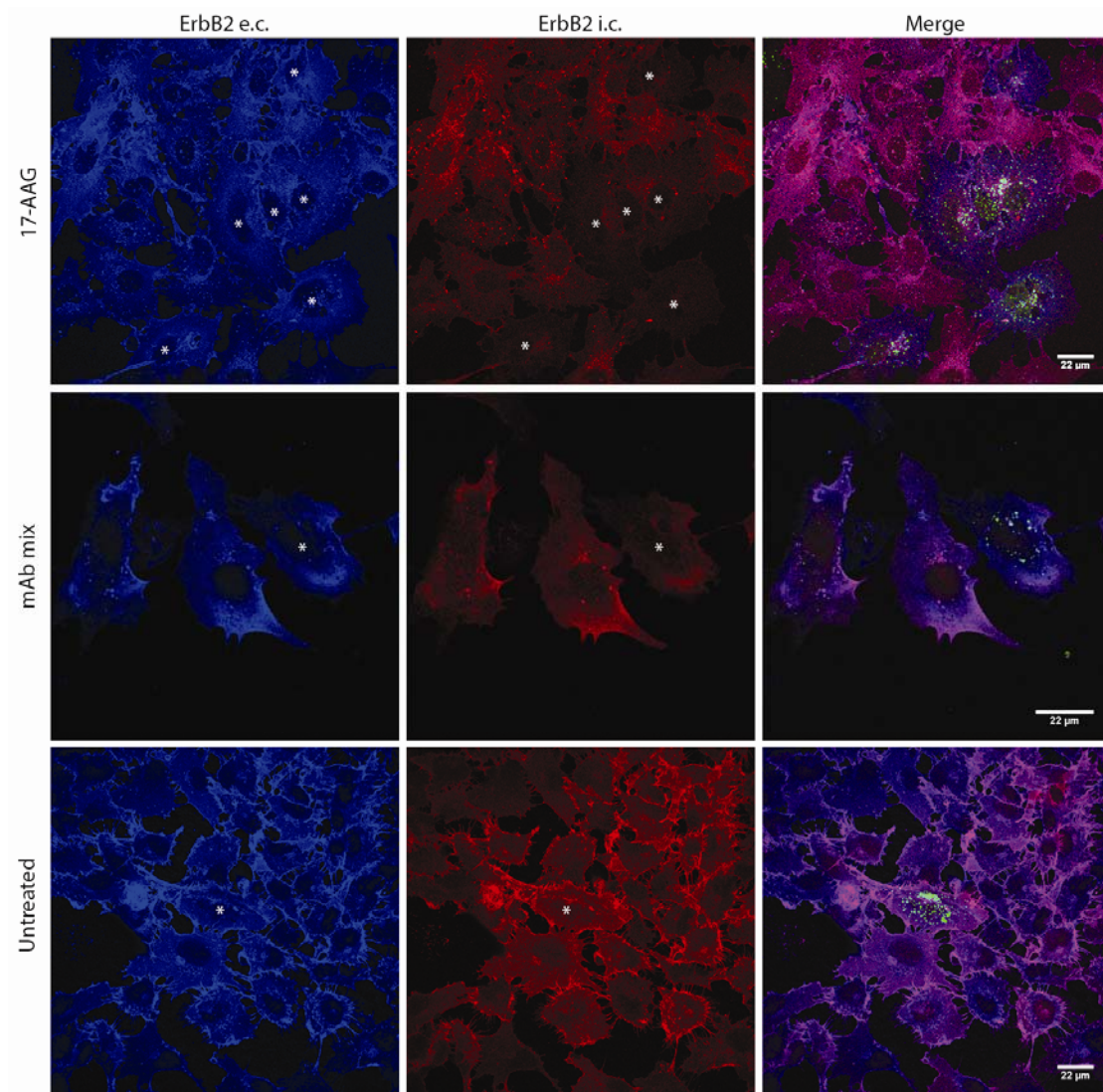


Figure 15: Inhibition of dynamin-dependent endocytosis causes retention of ErbB2 on the plasma membrane upon incubation with the mAb mix, but induces degradation of the intracellular region of ErbB2. PAE.ErbB2 cells transfected with GFP-TULA were incubated with mAb mix or 17-AAG for 4 hours. They were then labelled with mouse α -ErbB2 (extracellular region) antibody and rabbit α -ErbB2 (intracellular region) antibody, followed by Alexa649 α -mouse antibody and Rhodamine Red-X α -rabbit antibody. TULA was marked by a GFP tag. Cells that are transfected are marked with a star (*).

4.10 ErbB2 is phosphorylated upon treatment with α -ErbB2 antibodies

To investigate whether antibody-induced internalization of ErbB2 involved phosphorylation of the receptor, PAE.ErbB2 cells were incubated with mAb1, mAb2, mAb mix, trastuzumab, pertuzumab, trastuzumab and pertuzumab, or 17-AAG for 1 hour. They were then lysed, subjected to SDS-PAGE and analyzed by western blotting. Three tyrosine phosphorylation sites were investigated; pY1112, pY1221/1222, and pY1248 (Figure 17-19). The total level of ErbB2 under the same conditions was also checked (Figure 16). The Symphogen α -ErbB2 antibodies caused an increase in phosphorylated ErbB2 in all sites but pY1248. Trastuzumab and pertuzumab also caused increased phosphorylation, particularly pertuzumab (Figure 17-19).

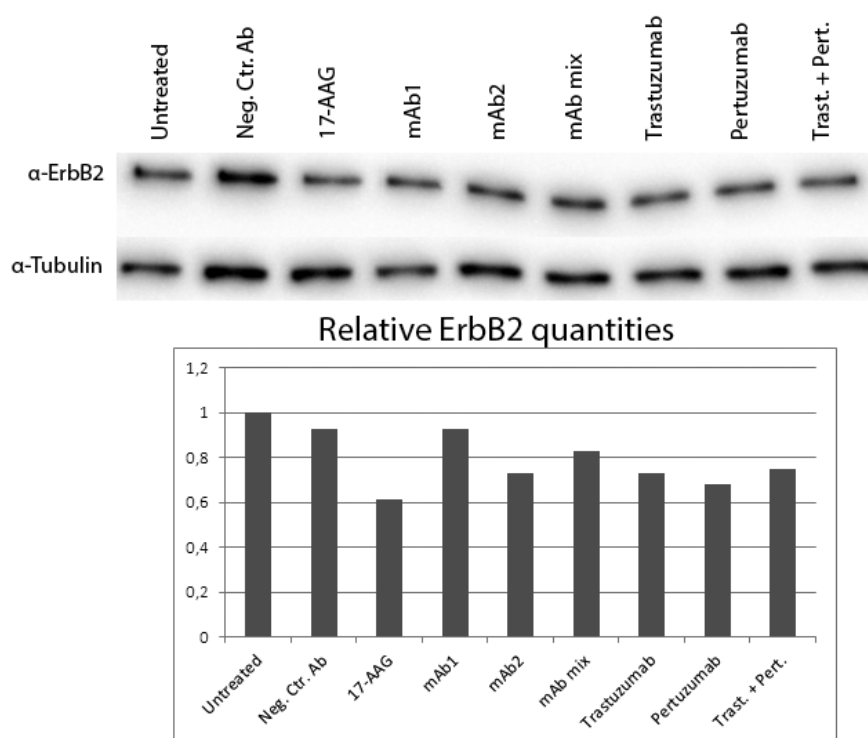


Figure 16: PAE.ErbB2 cells were incubated under the conditions shown for 1 hour and then lysed and analysed by western blotting. ErbB2 was labelled with a mouse α -ErbB2 antibody, followed by an HRP-conjugated α -mouse secondary antibody. ErbB2 quantities were normalized using tubulin as loading control.

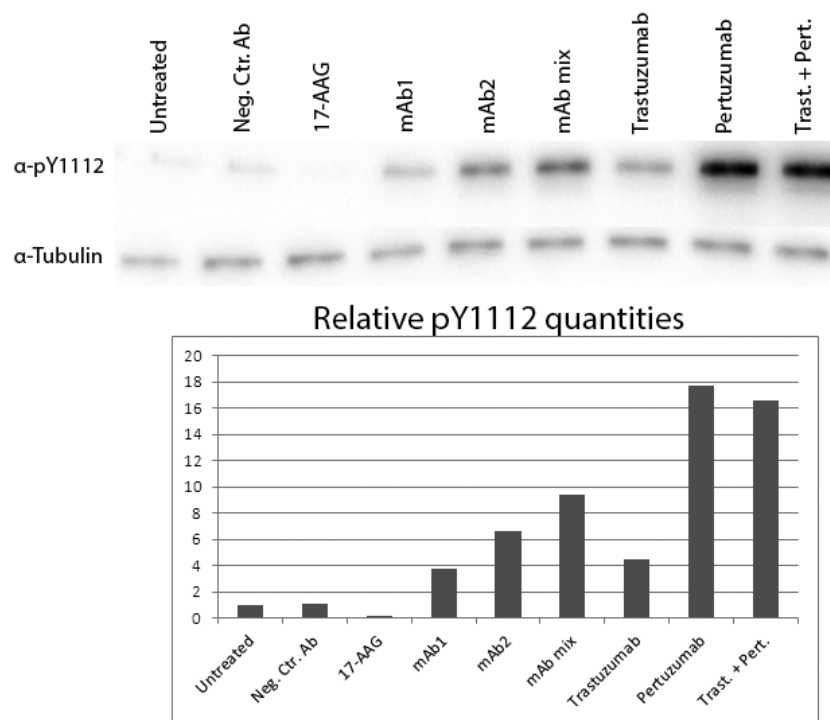


Figure 17: PAE.ErbB2 cells were incubated under the conditions shown for 1 hour and then lysed and analysed by western blotting. The phosphotyrosine pY1112 was labelled with a rabbit α -pY1112 antibody followed by an HRP-conjugated α -rabbit secondary antibody. Phosphorylated ErbB2 quantities were normalized using tubulin as loading control.

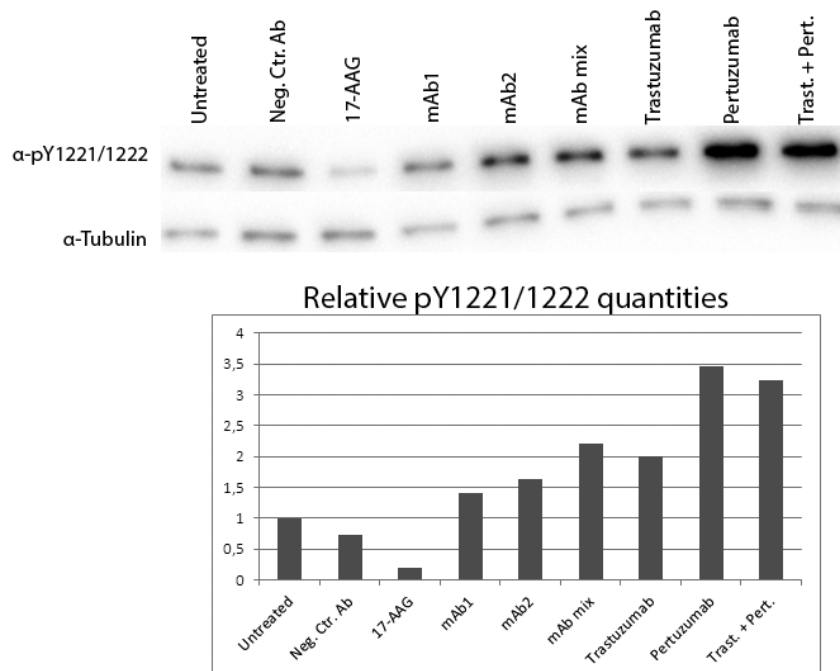


Figure 18: PAE.ErbB2 cells were incubated under the conditions shown for 1 hour and then lysed and analysed by western blotting. The phosphotyrosine pY1221/1222 was labelled with a rabbit α -pY1221/1222 antibody followed by an HRP-conjugated α -rabbit secondary antibody. Phosphorylated ErbB2 quantities were normalized using tubulin as loading control.

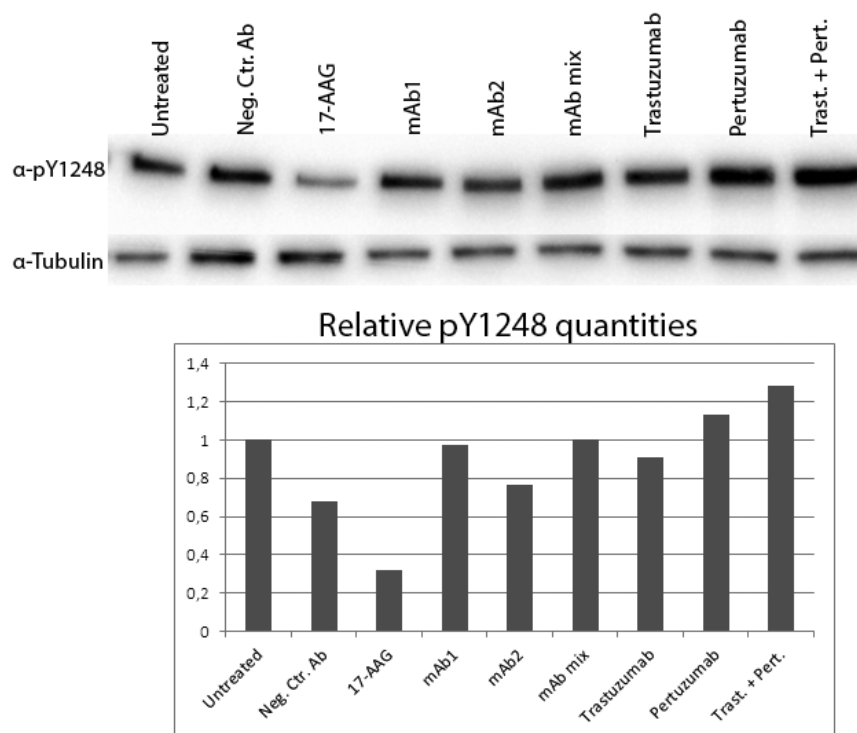


Figure 19: PAE.ErbB2 cells were incubated under the conditions shown for 1 hour and then lysed and analysed by western blotting. The phosphotyrosine pY1248 was labelled with a rabbit α -pY1248 antibody followed by an HRP-conjugated α -rabbit secondary antibody. Phosphorylated ErbB2 quantities were normalized using tubulin as loading control.

4.11 ErbB2 is ubiquitinated upon treatment with α -ErbB2 antibodies

Since it has been shown that ubiquitination of ErbB2 can induce internalization, an experiment was conducted to find out if treatment with antibodies induces ubiquitination. PAE.ErbB2 cells were treated with mAb mix, trastuzumab and pertuzumab, or 17-AAG for one hour. The cells were then lysed and ErbB2 was isolated by immunoprecipitation. The isolated sample, as well as whole cell lysate, was then subjected to SDS-PAGE and analyzed by western blotting. Ubiquitinated ErbB2 was identified by labeling with a mouse α -ubiquitin antibody. The membrane was then stripped and labeled with a mouse α -ErbB2 antibody to check how much ErbB2 had been isolated. The analysis showed that both the Symphogen α -ErbB2 antibodies and the trastuzumab and pertuzumab combination induced ubiquitination of ErbB2. However, not as strongly as the 17-AAG positive control.

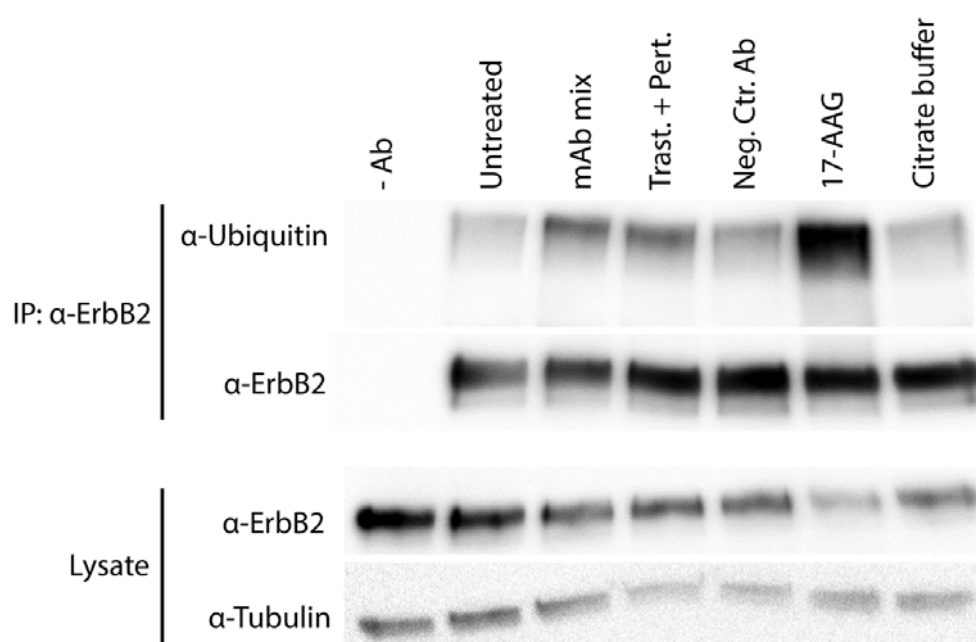


Figure 20: ErbB2 is ubiquitinated upon treatment with Symphogen α -ErbB2 antibodies.

PAE.ErbB2 cells were incubated under the conditions shown for 1 hour and then lysed. Immunoprecipitation with an α -ErbB2 antibody was used to isolate ErbB2 from the lysate. Immunoprecipitation without an α -ErbB2 antibody was carried out on one sample as a negative control. The isolated ErbB2 was analysed with regards to ubiquitination by western blotting using an α -ubiquitin antibody. A portion of the whole cell lysate was analysed by western blotting using an α -ErbB2 antibody to see how much total ErbB2 there was versus how much was isolated. An HRP-conjugated α -mouse secondary antibody was used to generate the signal.

5 Discussion

The present thesis has concentrated on the effect the Symphogen α -ErbB2 antibodies have on internalization and degradation of ErbB2 in comparison to trastuzumab and pertuzumab which are both therapeutic α -ErbB2 antibodies that are currently in clinical use. It also sought to find out whether or not incubation with the Symphogen α -ErbB2 antibodies induced phosphorylation and ubiquitination and to what extent internalization of ErbB2 induced by the Symphogen α -ErbB2 antibodies was dynamin-dependent.

It has previously been shown that a combination of trastuzumab and pertuzumab induces internalization of ErbB2 (61). The immunocytochemical experiments where NCI-N87 and PAE.ErbB2 cells were analyzed by confocal microscopy clearly showed internalization and localization to EEA1-positive endosomes of ErbB2 after 4 hours of incubation with the mAb mix (Figure 4, 5, 9, 10). The effect of the mAb mix appeared to be greater than that of the trastuzumab and pertuzumab combination. This was observed in all of the confocal microscopy experiments and although this needs to be verified by quantitation, the results strongly indicate that the mAb mix is likely a better inducer of ErbB2 internalization than trastuzumab and pertuzumab.

Furthermore, western blot analysis of ErbB2 degradation in NCI-N87 cells after they had been incubated for 24 hours with mAb1, mAb2, mAb mix, trastuzumab, pertuzumab, or a combination of trastuzumab and pertuzumab, showed good effect on ErbB2 degradation in cells treated with both antibody combinations (Figure 6). This shows that ErbB2 is degraded after being internalized, rather than being recycled back to the plasma membrane. Hughes et al. (61) did not observe ErbB2 degradation after treatment with trastuzumab and pertuzumab, but this could be because they incubated the cells for 5 hours, while the degradation observed in this thesis was after incubation for 24 hours. We have not seen degradation of ErbB2 after incubation with mAb mix for 5 hours either. Quantification of ErbB2 levels after antibody-induced degradation indicates that the mAb mix seems to cause a more effective degradation than the trastuzumab and pertuzumab combination. While this depends on statistical analysis to be verified, it supports the immunofluorescence data which indicates that the mAb mix is more effective than trastuzumab and pertuzumab. The effect on ErbB2 degradation in cells treated with individual antibodies was not as pronounced, lending support to the notion that combination treatments with several antibodies are more

effective. This could be explained by the possibility that the antibodies causes cross-linking between the receptors, which could lead to downregulation (63), although looking at ErbB2 on the plasma membrane by electron microscopy did not reveal the same amount of clustering (unpublished results) as was observed for EGFR in cells treated with cetuximab and a secondary α -human IgG antibody (68).

As described in the introduction there are several endocytosis pathways, some of which are dynamin-dependent. Experiments were conducted to find out if antibody-induced endocytosis depends on dynamin. When dynamin was knocked down with siRNA in NCI-N87 cells, it did not appear to have an effect on antibody-induced ErbB2 degradation (Figure 7) compared to the controls. This result could indicate that the internalization was not dynamin-dependent. However, the 17-AAG positive control, which is known to induce clathrin-dependent (and therefore dynamin-dependent) endocytosis of ErbB2, did not appear to be affected either. A smaller truncated protein consisting of ErbB2's extracellular and transmembrane regions is created when internalization is inhibited because the intracellular region is split off and degraded (30). Upon further examination, blotting for ErbB2 lower on the membrane, this fragment was found in the lysate of all the cells, but only the dynamin deficient cells treated with 17-AAG showed a stronger band than the untreated control (Figure 8). This indicated that the degradation of ErbB2 induced by the 17-AAG positive control was somewhat inhibited, though not completely blocked, when dynamin was knocked down, which is how it should be. The amount of truncated ErbB2 fragment was not found to be increased in the cells incubated with mAb mix or trastuzumab and pertuzumab, and this could indicate that their degradation was not dynamin-dependent. However, it should be noted that the siRNA did not produce a full knockdown of dynamin. While there was much less dynamin in the knockdown cells than in the controls, it is possible that what dynamin was left was sufficient for effective antibody-induced (but not 17-AAG-induced) internalization of ErbB2. It has previously been shown that it is difficult to get enough dynamin depletion to inhibit dynamin-dependent endocytosis (69). Based on this experiment it is therefore not possible to conclude whether the antibody-induced internalization of ErbB2 is dynamin-dependent or not.

Actually, the results of the K44A dynamin- and TULA transfection experiments give reason to believe that internalization of ErbB2 is dynamin-dependent. The GTPase-deficient mutant K44A dynamin and the Cbl-interacting protein TULA are both

inhibitors of dynamin-dependent endocytosis (66, 70). When PAE.ErbB2 cells were transfected to express either of these proteins, immunocytochemically labeled and analyzed with a confocal microscope after incubation with mAb mix or trastuzumab and pertuzumab, they had less vesicular ErbB2 than untransfected cells, although internalization was not completely inhibited (Figure 12). The amount of internalized ErbB2 in a transfected cell appeared to be inversely proportional to how high the expression of K44A dynamin or TULA was. This indicates that dynamin is involved in antibody-induced internalization of ErbB2. Furthermore, incubation of TULA transfected, and thus dynamin-inhibited, cells with the mAb mix was found to cause reduced labeling of the intracellular region of ErbB2 while labeling of the extracellular region seemed to remain at normal levels on the plasma membrane (Figure 14, 15). This indicates that the intracellular region of ErbB2 splits off or is degraded at the plasma membrane, as previously described in the case of inhibition of GA-induced internalization (30), when antibody-induced internalization is inhibited. Altogether these results favor the idea that antibody-induced internalization of ErbB2 is, at least to a certain extent, dynamin-dependent.

The earlier studies done by this group have shown that the degradation of the intracellular region upon inhibition of GA-induced internalization of ErbB2 is dependent on proteasomal activity, which indicates that this could happen as the result of ubiquitination of the receptor (30). Furthermore, it has been shown that antibodies induce ubiquitination of ErbB2 and that this depends on phosphorylation of pY1112 and recruitment of the ubiquitin ligase Cbl (71). Experiments were therefore conducted to examine the phosphorylation state of three ErbB2 phosphotyrosine sites, Y1112, Y1221/1222, and Y1248, after incubation with antibodies (Figure 16-19). Tyrosines 1112 and 1221/1222 showed increased phosphorylation after incubation with mAb mix or trastuzumab and pertuzumab. Pertuzumab in particular seemed like a potent inducer of phosphorylation. The mAb mix induced more phosphorylation than mAb1 or mAb2 by themselves. Viewed together with the finding that mAb mix is more effective at inducing degradation of ErbB2 than the individual antibodies, this could indicate that phosphorylation of ErbB2 is important to that process. Tyrosine 1248 did not show significantly increased phosphorylation, but was to a high extent also phosphorylated in untreated control cells, which may explain why there was no further increase in phosphorylation upon antibody treatment. The fact that antibody-treatment induces phosphorylation could indicate that they cause dimerization or

cross-linking. In the case of pertuzumab, which inhibits ErbB2 dimerization and should therefore block autophosphorylation, the strong phosphorylation it induces is odd and difficult to explain. Perhaps it induces cross-linking that brings the ErbB2 proteins close enough together to phosphorylate each other.

Because of the previously mentioned studies which show that ubiquitination is sufficient for ErbB2 internalization (33) and that phosphorylation of Y1112 is important to ubiquitination (71), the finding that mAb mix and trastuzumab and pertuzumab causes phosphorylation of Y1112 prompted an experiment to see if they also induce ubiquitination of ErbB2 (Figure 20). ErbB2 from cells that had been incubated with antibodies for 1 hour were isolated by immunoprecipitation and then examined for covalently attached ubiquitin. It was found that both mAb mix and trastuzumab and pertuzumab induced ubiquitination of ErbB2. This indicates that ubiquitination could be the cause of the degradation of the ErbB2 intracellular region when endocytosis is inhibited. Or that ubiquitination causes internalization.

Ubiquitination of the receptor has been shown to be sufficient for internalization and degradation of ErbB2 (33).

In conclusion, the results of this thesis show that the Symphogen α -ErbB2 antibodies induce internalization and degradation of ErbB2. The antibodies are more effective in combination than by themselves and they appear to be more efficient than trastuzumab and pertuzumab. Furthermore, it shows that the antibody-induced endocytosis of ErbB2 most likely is dynamin-dependent, at least to a certain degree, and causes both phosphorylation and ubiquitination of the receptor. One could speculate about whether phosphorylation and ubiquitination is important to the antibody-induced endocytosis of ErbB2. To make a final conclusion, however, further verification of these data are needed. Quantitative data about downregulation from the plasma membrane could be gotten by flow cytometry. Use of kinase inhibitors could tell us more about whether or not phosphorylation is necessary for ubiquitination and for internalization. Using siRNA to knock down clathrin could tell us more about the endocytic pathway used. However, it could be difficult to get clear data about this as it is possible that more than one endocytic pathway is involved in the endocytosis of ErbB2. This has been shown to be the case for endocytosis of EGFR which uses both clathrin-dependent and -independent pathways. It is probable that the antibodies cause cross-linking of ErbB2s at the plasma membrane which could induce dynamin-independent macropinocytosis, as has been shown to be the case for EGFR (68).

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